THE EFFECT OF SELENIUM ON THE METABOLISM OF RUMEN BACTERIA *IN VITRO*.

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Sodium selenate was added to rumen fluid and artificial saliva mixtures which were incubated with either a hay or starch substrate. Using a starch substrate, selenate up to 0.3mg/*l* increased bacterial protein synthesis and volatile fatty acid (VFA) production and reduced the pH of incubation mixture. Selenate levels above 0.3mg/*l* had the opposite effect and also altered the proportions of VFA produced. With a hay substrate, selenate had little effect at low concentrations, but at higher concentrations similar effects were noted with VFA production and protein synthesis declining. The % of hay substrate digested was also reduced at selenate concentrations above 3mg/*l*.

With a starch substrate the stimulatory effects of selenium on bacterial metabolism were thought to be due to selenium promoting bacterial multiplication. The lack of significant increases in VFA production and protein synthesis with hay substrate may have been a result of much of the selenate being reduced by the bacteria to unavailable forms.

KEYWORDS : selenium; selenate; rumen bacteria; protein synthesis; volatile fatty acids.

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CHAPTER 1

INTRODUCTION

Selenium, long known to be toxic to living organisms (Franke, 1934), has been considered an essential element since the classic studies on rats by Schwarz and Foltz in 1957. Soon afterwards it was shown to prevent nutritional muscular dystrophy (NMD) in calves (Muth, Oldfield, Remmert and Schubert, 1958). As about six million hectares of farmland in New Zealand are considered to be deficient in selenium (Millar, 1983) and its deficiency can cause infertility, NMD, lowered production and even death in sheep and cattle (Stevens, 1981), its importance as a trace element for ruminants has long been recognised in this country.

A minimum requirement for selenium in ruminant diets has been established at around 0.03mg/kg DM (Andrews, Hartley and Grant, 1968; Gardiner, Armstrong, Fels and Glencross, 1962) which, if not met by the diet, has traditionally been provided by periodic drenching, or more recently by the top-dressing of pastures with selenium prills. Recent evidence has suggested that the former dosing method has only short-term benefits (Taylor, 1984) and the latter entails an uncertain and somewhat uncontrolled intake. This has led to the development of glass or metal pellets which are placed directly in the rumen to provide a slow release of selenium over long periods (Taylor, 1984).

As any compound in the rumen is exposed to microbial interaction, a widespread use of ruminal selenium pellets in the future makes research on selenium's effects on microbial activity all the more important.

Although experiments on selenium's effect on non-rumen bacteria began as early as the 1950s and selenite was included in a group of elements added to pure strains of the rumen bacterium *Micrococcus lactilyticus* in 1962 (Woolfolk and Whiteley, 1962), it was not until 1968 that researchers began to examine selenium in the rumen and its effects on the metabolism of the rumen microorganisms.

The aim of these experiments is to extend the previous findings of the effect of selenium on rumen bacteria, explain the conflicting results obtained by other researchers in this field, and indicate an appropriate selenium concentration to optimise rumen bacterial activity.

CHAPTER 2

REVIEW OF THE LITERATURE

2.1 MICROBIOLOGY OF THE RUMEN

While an extensive review of the microbial population is unnecessary as several good reviews are available (Hungate, 1966a; Bryant, 1959; Bryant, 1970; Hobson, 1971), an adequate understanding is necessary prior to a discussion of rumen metabolic pathways and the effect of selenium on the latter.

2.1.1 Protozoa and Bacteria

The volume of microorganisms in strained rumen liquor is around 3.6% v/v comprising about 50% ciliates and 50% small bacteria (Warner, 1962), but the number and species of these is dependent on a number of factors (Blaxter, 1967).

Most rumen protozoa are ciliates which can grow in the presence of billions of accompanying bacteria. They are beneficial to the host animal in that they increase VFA production (Howard, 1959) and the digestibility of organic matter (Bryant, 1970). Hungate (1955) estimated that protozoa may account for about 20% of the protein requirement, and a similar amount of the acid fermentation products available to the animal.

In general, organisms with small individual cell volumes are much more active than those with large cell volumes. Thus, although the total volume of small bacteria

is about the same as that of the ciliates (Warner, 1962), their metabolic activity is undoubtedly much greater (Bryant, 1970) and therefore their importance in the rumen. This is why almost all of the work done on selenium and rumen microorganisms has concentrated on the bacterial population. The total number of bacteria per gram is usually in the range of 15 (Bryant and Burkey, 1953) to 50×10^{12} (Doetsch and Robinson, 1953). Bryant (1959) mentioned 29 genera and 63 species of bacteria in his review, most utilising one or more types of the major dietary carbohydrates as an energy source for growth. Those that do not, utilise the simpler hydrolytic products of these or major end products of carbohydrate metabolism, such as lactate, formate or hydrogen. In addition, many species have a requirement for branched-chain VFA (Bryant and Robinson, 1961).

Although this complex mixture of microorganisms of different metabolic functions is present in the rumen of animals maintained on most dietary regimes, there is a relatively small number of metabolic pathways leading to the end products (Hobson and Wallace, 1982). The mixed population ensures that only those end products of fermentation accumulate which are not susceptible to further breakdown. They are the volatile fatty acids (VFA), methane and CO_2 (Bryant, 1959; Hungate, 1960).

2.1.2 Stability and Homogeneity of the Rumen Microbial Population in Relation to Collection of Rumen Fluid

When *in vitro* incubations are performed at sporadic

times over a long period, it is important that the rumen fluid used for these experiments be of as consistent a microbial composition as possible. This can be achieved by minimising the transient changes that occur in the microbial population resulting from constantly occurring shifts in rumen ecological niches.

The two most important factors influencing the numbers and kinds of microorganisms within the rumen, are the nature of the ration (Elsden, 1945) and the time interval since feeding (Moir and Williams, 1950). As about ten days are needed for the microbial population to restabilise following a change of diet (Warner, 1962), the ruminant should be maintained on a diet of unvarying composition to avoid alterations to the rumen population.

Although Hungate (1966b) emphasised the advantage of keeping feed continuously available to the animal to maintain maximum growth and productivity of the microbial population, a short period of starvation is required before collection of rumen fluid to make the removal procedure easier.

As there is a higher concentration of microbes in whole digesta than in the lower, liquid layer of freshly collected rumen liquor (Hungate, 1966c), because twice as many bacteria are associated with the particles of rumen fluid (Warner, 1962), the straining of rumen liquor to remove dietary originated fibre also removes many of the rumen microbes. A period of animal starvation lessens the presence of dietary originated fibre but has little effect

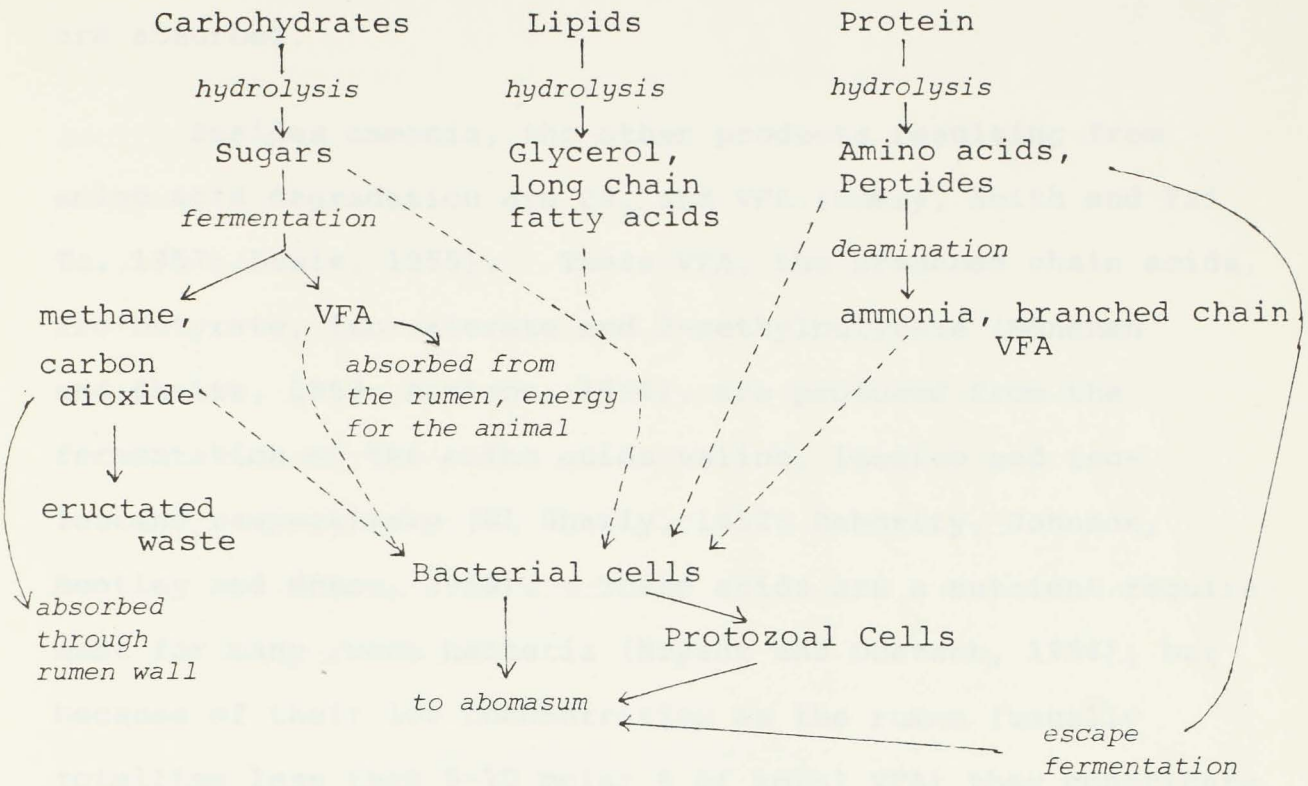
on the composition and concentration of bacteria (Gilchrist and Kistner, 1962). Although total bacterial numbers may decline, the concentration remains similar due to the absence of dilution by saliva and roughage with feeding (McAnally, 1943; Kistner, Gouws and Gilchrist, 1962). The end result is a more liquid rumen fluid and subsequently less loss of bacteria when the small amount of dietary originated matter is removed by straining.

Although a fistula does not affect the activity of the microorganisms (Hungate, 1966b), the removal of a significant amount of rumen contents affects the metabolism of the remaining microrganisms (Warner, 1966). Therefore long intervals are necessary between regular fluid collections to enable complete recovery of the rumen population.

2.2 METABOLISM OF DIETARY COMPONENTS IN THE RUMEN

2.2.1 Introduction

The major foodstuff components in the ruminant diet, hexose polymers (cellulose, starch and fructosans), pentose polymers (mostly xylan), protein and small quantities of fat, are all initially exposed to the fermentative activity in the rumen. Fermentative digestion is advantageous for substrates that cannot be digested by the animal's own enzymes or that have poor nutritional qualities, and in effect, the rumen microorganisms modify the food of the ruminant before normal digestion proceeds later in the alimentary tract (Figure 1).



------→ incorporation into bacterial cells, in part at least

——→ metabolic pathways

Figure 1 The principal reactions in the rumen (Hobson and Wallace, 1982)

2.2.2 Protein Metabolism

The importance to the animal of the proteolytic activity possessed by both bacteria and protozoa (Allison, 1969) is that the deamination of amino acids (Annison, 1956) is accompanied by the formation of ammonia (Pearson and Smith, 1943). This ammonia, together with that derived from non-protein dietary nitrogen, may either be absorbed directly through the rumen wall or converted to microbial protein (Lewis, 1961). This microbial protein and any undigested protein, pass unchanged to the abomasum (Blackburn

and Hobson, 1960) and serve as a source of amino acids which are absorbed.

Besides ammonia, the other products resulting from amino acid degradation are $C0_2$ and VFA (Emery, Smith and Fai To, 1957; Lewis, 1955). These VFA, the branched chain acids, iso-butyrate, iso-valerate and 2-methylbutyrate (Menahan and Shultz, 1963; Annison, 1954), are produced from the fermentation of the amino acids valine, leucine and iso-leucine respectively (El Shazly, 1952; Dehority, Johnson, Bentley and Moxon, 1958). These acids are a nutrient requirement for many rumen bacteria (Bryant and Doetsch, 1954), but because of their low concentration in the rumen (usually totalling less than 5-10 molar % of total VFA) they contribute little to the ruminant energy needs. Warner (1964) concluded that as a source of energy to the animal, proteins provide no more than about 10% of the total requirements.

2.2.3 Fat Metabolism

Fats constitute a negligible percentage of usual ruminant feeds and, apart from hydrolysis and hydrogenation, are not further broken down in the rumen (Wright, 1961b). Dietary fat together with microbial lipids, do not provide more than about 10% of the ruminant's energy needs (Warner, 1964), and will not be discussed here further.

2.2.4 Carbohydrate Metabolism

Of all the dietary components of normal ruminant feeds, the carbohydrates are present by far in the largest quantities, and because of this, and the fact that their conversion to VFA yields substantial ATP, they must be considered as the

greatest source of biologically useful energy.

Whittenbury (1981), in his review of bacterial nutrition, characterises the rumen microbial processes as a succession of fermentations (outlined in figure 2), and this review will be based on his classifications: (1) the breakdown of cellulose, pectins and hemicelluloses by bacteria with the necessary extra-cellular enzymes, (2) the fermentation of the monosaccharide products by a second group of bacteria to the VFA, hydrogen and CO_2, (3) and finally the conversion of CO_2 and H_2 to methane by the methanogenic bacteria.

2.2.4.1 Polysaccharide Breakdown

This multistage process is complex, and other enzymes and intermediates may occur in addition to the well documented ones mentioned below.

It has been shown that 70-90% of all forms of cellulose became soluble within three days of entering the rumen (Halliwell, 1957) and that 70% of the digestible cellulose in a fodder was broken down in the rumen (Gray, 1947). The cellulolytic bacteria responsible for this produce extracellular enzymes (Dehority, 1968) which degrade cellulose to oligosaccharides and finally to cellobiose which is then split to glucose (Reese, Siu and Levinson, 1950) and glucose-1-phosphate (Ayers, 1959).

Following starch breakdown to maltose by the amylases (Hobson and MacPherson, 1952) of a variety of bacteria and protozoa (Mould and Thomas, 1958), the maltose is cleaved

by maltases and iso-maltases (Bailey and Howard, 1963), with the formation of glucose.

Hemicelluloses contain a diverse range of compounds, and are hydrolysed to the simpler oligosaccharides in a series of steps by microorganism enzymes (Thomas, 1960) and finally to pentose sugars. Pectin is rapidly hydrolysed by enzymes produced by both bacteria and protozoa (Wright, 1961a) to galacturonic acid, and fructosans are degraded to fructose mostly by the protozoal enzymes (Thomas, 1960).

Before fructose and the pentose sugars can be utilised in the second stage of the metabolic chain, they must first be changed into hexose sugars, which is accomplished by the conversion of six pentose molecules to five hexose monophosphate molecules (Hungate, 1966e). Once all dietary carbohydrates have been degraded to the hexose monophosphate, glyceraldehyde-3-phosphate, breakdown is via the Embden-Meyerhoff pathway (Baldwin, Wood and Emery, 1963) to produce two pyruvate molecules, 3 ATP and 2 (2H).

2.2.4.2 VFA Production Pathways

100 years ago Tappeiner (1882; 1883; 1884) showed that the microorganisms in the rumen fermented cellulose to produce the end products methane, CO_2, and VFA. Since his identification of formic, acetic, propionic and n-butyric acids, other workers have identified branched and longer chained

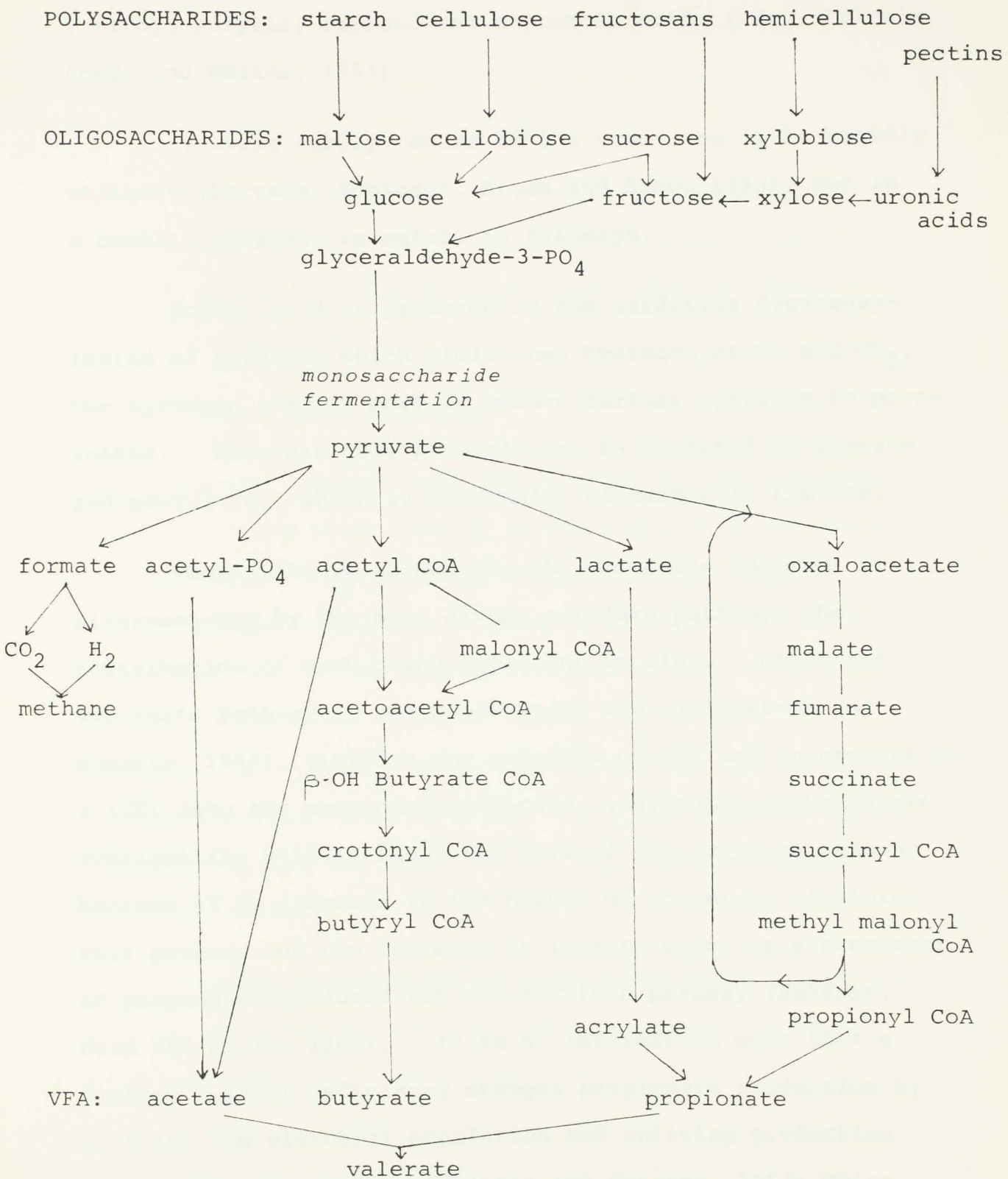


Figure 2: Carbohydrate fermentation pathways. (Adapted from Barnett and Reid (1961), Hungate (1966e) and Leng (1969).)

VFA in the rumen viz. iso-butyric, valeric, iso-valeric, 2 methyl butyric, caproic and heptanoic acids (Gray, Pilgrim, Rodda and Weller, 1951).

Pyruvate rarely occurs in the rumen, as it is rapidly utilised (Doetsch, Robinson, Brown and Shaw, 1953), but is a central compound in metabolic pathways.

Acetic acid is produced by the oxidative decarboxylation of pyruvate which yields two hydrogen atoms and CO_2, the hydrogen used in part to reduce further pyruvate to propionate. Alternatively pyruvate may be degraded to formate and acetyl-PO_4, which is eventually converted to acetate.

Propionate is formed via the succinate pathway, or alternatively by the more direct acrylate pathway, the contribution of each being dependent on diet. While the succinate pathway is the predominant one (Blackburn and Hungate, 1963), yielding one molecule of ATP and incorporating 2 (2H) into the propionate molecule, increasing carbohydrate availability will increase the role of the acrylate pathway because of an increase in the number of organisms utilising this pathway and the increase in lactate which is a precursor of propionate produced via the acrylate pathway (Baldwin, Wood and Emery, 1963). It is of interest to note that a dietary sulphur deficiency changes propionate production by altering the microbial population and shifting production to the acrylate pathway (Whanger and Matrone, 1965; White, Steel, Leng and Luick, 1969).

Butyrate is formed by the condensation of two molecules of acetic acid via the malonyl pathway, or alternatively

from pyruvate, giving rise to acetyl CoA (Barker, 1961). In the former pathway 2 moles of ATP are required for the formation of butyrate, compared with the one mole involved in the condensation of acetyl CoA molecules.

Higher normal acids are formed in the rumen by the condensation of the lower acids with a 2-carbon compound in equilibrium with acetic acid (Gray, Pilgrim, Rodda and Weller, 1951; 1952).

2.2.4.3 Methanogenesis

Formate derived from the cleavage of the pyruvate molecule, is oxidised rapidly in the rumen with the formation of hydrogen and CO_2 (Baldwin, Wood and Emery, 1963). There are two groups of anaerobic bacteria involved in the formation of methane, which can reach production levels of 50 litres per day in the sheep (Blaxter, 1967). They are the non-methanogenic organisms producing formate and hydrogen, and the methanogenic bacteria which, using the products of the former group for the reduction of CO_2, produce methane (Hungate, 1966c).

Formate is estimated to contribute about 18% of rumen methane via conversion to CO_2 and H_2 (Hungate, 1966e) Other precursors include alcohols and VFA, but of these, only acetate is thought to be significant under certain conditions (Isaacson, Hinds, Bryant and Owens, 1975).

The formation of methane is considered to be a wasteful process in terms of energy utilisation, since the proportion of gross energy in the food that is converted to methane

(8-10%) is expelled as gas and lost to the animal (Wolfe, 1972), and considerable effort has been devoted in many laboratories to prevent this energy loss (Chalupa, 1977). As practically all the methane formed in the rumen is produced via CO_2 reduction, with H_2 serving as the source of electrons (Bryant, 1965), the diversion of hydrogen from methane producing pathways is an important method of controlling methanogenesis. Any hydrogen produced is rapidly utilised (Carrol and Hungate, 1955) and is rarely detected in the rumen contents (Opperman, Nelson and Brown, 1959). Its production from hexose is dependant on the particular pathway utilised:

$$\text{hexose} + 2H_2O \longrightarrow 2 \text{ Acetate} + 2CO_2 + 4H_2$$

$$\text{hexose} + 2(2H) \longrightarrow 2 \text{ Propionic} + 2H_2O$$

$$\text{hexose} \longrightarrow \text{Butyrate} + 2CO_2 + 2H_2$$

Chalupa (1977) concluded that the metabolicaly useful energy recovered in the VFA can be increased by enhancing the production of propionate and to a lesser extent butyrate, at the expense of acetate production.

2.3 SELENIUM METABOLISM IN THE RUMEN

2.3.1 Introduction

In non-ruminants selenium is mainly excreted in the urine (Butler and Peterson, 1961). In ruminants however, the elimination of much of the selenium in the faeces, rather than the urine (Butler and Peterson, 1961), was thought by Hungate (1966d) to be a result of the microbial

activity in the rumen. The early work on selenium was done with non-rumen microorganisms, and provided a reference for research on the metabolism of selenium by rumen bacteria. This latter work was initially concerned with selenium incorporation into bacterial protein and later on the effects of selenium on overall rumen metabolism. However, an understanding of the earlier studies on selenium is necessary to understand the effects of selenium in the rumen.

2.3.2 Microbial Selenoenzymes

That selenium was essential in *E.coli* for the activity of the enzyme formate dehydrogenase (FDH) was first shown by Pinsent in 1954, but it was not until several years later that the work was followed up and several observations established that selenium was metabolised to organic compounds and incorporated into bacterial protein (Cowie and Cohen, 1957; Blau, 1961; Tuve and Williams, 1961). Later, workers found that selenium was incorporated as a constituent of FDH in *E.coli* and other bacteria (Enoch and Lester, 1972; Shum and Murphy, 1972; Andreesen and Ljungdahl, 1973).

Where selenium is present in a precisely specified location and participates as an essential enzyme component, Stadtman (1980a) defined the selenium dependent enzyme as a selenoenzyme. Since the identification of further selenoenzymes, including glycine reductase (Turner and Stadtman, 1973), Stadtman has discussed their function and importance in three detailed reviews (Stadtman, 1980a; 1980b; 1980c).

FDH is one of several components of the enzyme complex formate hydrogen lyase which produces CO_2 and H_2 from formate

(Gottschalk, 1979). Although FDH activity is widely distributed, only a few anaerobic bacteria have been investigated and are known to possess the selenium dependent form. The only rumen bacterium to have been studied in regard to a selenium dependent FDH enzyme was *Vibrio succinogenes* (Kröger, Winkler, Innerhofer, Hackenberg and Schägger, 1979) which utilises formate (Wolin, Wolin and Jacobs, 1961). Although it was similar to the selenium dependent FDH of *E. coli*, described by Enoch and Lester (1975), in that it was membrane bound, contained molybdenum and was a component of the electron transport chain, Kröger *et al.* (1979) concluded that it was not dependent on selenium for its activity.

In 1976 Hidiroglou and Lessard suggested that if the rumen microbial enzymes FDH and glycine reductase were selenium dependent, modifications found in rumen metabolism during selenium supplementation might be associated with the activities of these two enzymes. Glycine reductase reduces glycine to acetate and ammonia, but in the rumen glycine is only slightly deaminated (Lewis, 1955, and Lewis and Emery, 1962) and as the quantity of protein is usually small in most normal ruminant diets, alterations in the activity of glycine reductase would have minimal effect on overall rumen metabolism.

Of the naturally occurring selenoproteins, all but one are bacterial enzymes. Many rumen bacteria are able to utilise formate, and as it is an important compound in the rumen metabolic pathways, perhaps the identification of a selenium dependent FDH in rumen bacteria in future research

will elucidate the effects of selenium described by Hidiroglou and Lessard.

2.3.3 Selenium Substitution

As selenium occurs directly below sulphur in the periodic table, it resembles sulphur in many of its chemical properties, occurring in inorganic forms H_2SeO_3, H_2SeO_4 and H_2Se for example, which are analogues of the sulphur compounds H_2SO_3, H_2SO_4 and H_2S. There are also many biological molecules that contain sulphur for which selenium is able to substitute (Stadtman, 1974). Due to this close chemical similarity, Hidiroglou and Zarkadas (1976) suggested that the rumen microbial metabolism of selenium compounds might occur by the same pathways by which sulphur analogues are transformed. While several good reviews are available covering microbial sulphur metabolism (e.g. Shrift, 1967; Peck, 1970; Bray and Till, 1975), it is briefly outlined here, not only as a basis for discussion of experiments concerning the metabolic pathways of selenium, but also because of the effects of one element on the other during metabolism.

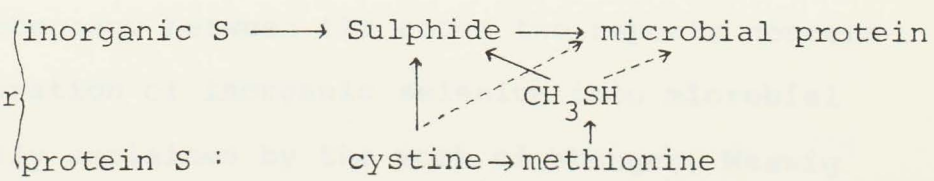
2.3.3.1 Sulphur Incorporation into Bacteria

It has long been known that ruminants have a requirement for sulphur (Thomas, Loosli, Williams and Maynard, 1951) and the sulphur containing amino acids, cyst(e)ine and methionine are important constituents of rumen bacteria (Johanson, Moir and Underwood, 1949). The ability of rumen microorganisms to utilise inorganic sulphur in the biosynthesis of amino acids, and subsequently proteins, has now been well

documented (Loosli, Williams, Thomas and Ferris, 1949 ; Block, Stekol and Loosli, 1951; Paulson, Baumann and Pope, 1968).

Ingested sulphate disappears from the rumen in 3-5 hours (Lofgreen, Weir and Wilson, 1953) and both cyst(e)ine and methionine are rapidly and extensively degraded in the rumen (Bird and Hume, 1970; Doyle and Moir, 1979). Although Anderson (1956) reported that the rumen microbiota utilised sulphate in preference to amino acid sulphur, both inorganic and organic dietary sulphur are degraded to free sulphide in the rumen (Lewis, 1954; Bird and Moir, 1972). This sulphide is used for *de novo* synthesis of sulphur amino acids (Allison, 1965) and is the main source of sulphur for cyst(e)ine and methionine synthesis (Hume and Bird, 1970), except where preformed sulphur amino acids are abundant in the diet and some direct bacterial incorporation of these may occur (McMeniman, Ben Ghedalia and Elliot, 1976). Other than some strains of *Bacteroides ruminicola* which require cyst(e)-ine and methionine (Pittman and Bryant, 1964) most rumen bacteria do not require organic sulphur compounds for growth, and direct incorporation of preformed amino acids was consid-ered by Allison (1969) to be quantitatively unimportant.

The established pathways of sulphur metabolism in the rumen are summarised below, although Gawthorne and Nader (1976) have suggested that sulphur may enter microbial protein by other routes.



The pathways of ruminal sulphur metabolism (Bray and Till, 1975).

2.3.4 Selenium Incorporation into Rumen Bacteria

The differences in faecal excretion of selenium between ruminants and non-ruminants (Wright and Bell, 1966), the microbial alteration of the form in which selenium is ingested (Hungate, 1966d) and the substitution of inorganic selenium for sulphur in the biosynthesis of amino acids in non-ruminal microorganisms (Blau, 1961; Tuve and Williams, 1961) prompted Hidiroglou, Heaney and Jenkins (1968) and Paulson, Baumann and Pope (1968) to undertake independent studies on the metabolism of selenium in the rumen.

The latter workers designed *in vitro* experiments in which rumen fluid was incubated with radioactive selenium or sulphur compounds and then precipitated with trichloroacetic acid to determine the amount of radioactivity associated with the protein fraction. Their results demonstrated that after incubation with selenomethionine, selenomethionine was recovered in the microbial protein. However, when ^{75}Se was added as selenite or selenate, there was very little biosynthesis of selenomethionine from inorganic selenium.

Data from both *in vivo* and *in vitro* experiments by the group of workers, Hidiroglou *et al.* (1968), showed that the metabolism and incorporation of inorganic selenium into microbial protein did indeed occur, predominantly via selenomethionine synthesis.

The discrepancy between the above two reports concerning the incorporation of inorganic selenium into microbial protein is partly explained by the work of Whanger, Weswig and Muth also published in 1968. They found that microorganisms incubated with ^{75}Se - selenite incorporated selenium

mostly as selenocystine, and as selenomethionine and selenocystine when incubated with selenomethionine. They also found that the inorganic forms of selenium were more readily converted to unavailable forms than organic selenium. Therefore two possibilities exist as to why Paulson *et al.* (1968) reported that inorganic selenium was not incorporated into bacterial protein. Selenium may have been converted to the unavailable forms or incorporated into selenocystine which was not detected because of its instability when released by the pronase digestion procedure used (Butler and Peterson, 1967). It is almost certain that the latter factor was the reason why selenocystine was not found when microorganisms were incubated with selenomethionine.

Hidiroglou *et al.* (1968) identified only small amounts of selenocystine using an acid hydrolysis extraction process, but Tuve and Williams (1961) found this method was frequently accompanied by extensive destruction and loss of organoselenium compounds and although they themselves recovered only selenomethionine, postulated the existence of selenocyst(e)ine.

Although results from all three authors were in agreement that selenomethionine was metabolised and incorporated into bacterial protein, confirmation of the results of Whanger *et al.* (1968) concerning the presence of significant levels of selenocyst(e)ine was not found until 1974 by Hidiroglou, Jenkins and Knipfel. In an *in vivo* study involving intraruminal administration of labelled selenomethionine, they found rumen liquor rapidly reached its maximum ^{75}Se specific activity. Up to six hours after dosing about 30% of the rumen liquor label was incorporated into bacterial

protein. Analyses of this protein showed 15-20% as selenomethionine and 8-12% as selenocystine, but the majority (40-50%) of the label was present as an unidentified compound. The presence of selenocystine was also confirmed by an *in vitro* incubation trial which indicated that it was formed by the metabolism of selenomethionine.

This incorporation is responsible for high levels of selenium in the protein of rumen microorganisms (Hidiroglou *et al.* 1968). Whanger *et al.* (1968) extracted microbes following incubation with selenite and on analysing the residual bacterial protein, found selenium concentrations ranging up to 50 ppm.

Two- to 78- fold increases of the selenium content of microorganisms over that in the diet have been noted (Whanger, Weswig, Muth and Oldfield, 1970; Whanger, Weswig and Oldfield, 1978).

Similar results have been reported by Martinez (1972) in bovine rumen microorganisms. Thus it would seem that ruminal microbes do not only incorporate selenium into their proteins, but also concentrate it within their cells.

2.3.4.1 The Mechanisms by which Selenium is Incorporated into Bacterial Protein

Although these mechanisms are unknown at present, at least in rumen bacteria, some of the researchers investigating the incorporation of selenium into rumen bacterial protein have suggested that direct incorporation of the intact selenoamino acids occurred. Considering the comments

of Stadtman (1980c) that this would require a specific codon for the selenoamino acid during polypeptide chain elongation, it is unlikely to occur where selenium is not an essential component of the protein. Alternatives suggested by Stadtman were :

1) modification of an existing amino acid in a polypeptide chain, e.g. addition of selenide to a cysteine residue already present in a protein, to form selenocysteine and

2) the addition of a selenoamino acid to the end of a polypeptide chain, then linking with a second polypeptide.

If both these mechanisms were operating in rumen bacteria it might explain the differences in the incorporation of organic and inorganic selenium, as the first alternative allows only the incorporation of inorganic selenium, and the second, selenoamino acids.

The first alternative, where inorganic selenium is incorporated into an existing protein, has been shown to occur in animal tissue proteins (Jenkins and Hidiroglou, 1971). In that instance the cystine content of a protein seemed to affect selenium incorporation because of selenotrisulphide (R-S-Se-S-R) formation. When ^{75}Se- $NaSeO_3$ reacted with cysteine and cystine in aqueous solution, Czauderna and Samochocka (1981) found selenodicystine (a selenotrisulphide) was formed within 15 minutes. As the majority of inorganic selenium, added by Paulson *et al.* (1968) to rumen fluid *in vitro*, was rapidly incorporated into the TCA insoluble fraction, but not enzyme dependant, it may have been incorporated as a selenotrisulphide.

More research is needed before the selenium incorporation pathways can be conclusively established, but as differences in the metabolism of selenium and sulphur in rumen bacterial incorporation studies have been reported (Paulson *et al.*, 1968; Hidiroglou *et al.*, 1968), the previously mentioned mechanisms would explain this.

2.3.5 The Effect of Sulphur on Selenium Metabolism

On addition of sulphate to a diet containing 0.01 mg selenite/kg, Muth, Schubert and Oldfield (1961) found a decrease in the weight gain of lambs and attributed it to an apparent interference of selenium by sulphur (because of their chemical similarities) creating a selenium deficient situation. Hintz and Hogue (1964) also found dietary sulphur could increase the clinical incidence of nutritional muscular dystrophy in lambs.

Additional dietary sulphur has since been shown to increase selenium excretion (White and Somers, 1977; Pope, Moir, Somers, Underwood and White, 1979). Pope *et al.* (1979) attributed this to the effects of sulphur on the rumen microbiota. Additional sulphur would not only increase its reduction to H_2S but also increase the amount of selenium reduced by similar bacterial metabolic pathways (Shrift, 1973) to H_2Se. The more unstable H_2Se could be either metabolised and reutilised by the bacteria or form insoluble metal selenides not able to be absorbed and utilised by the ruminant. Therefore, Pope *et al.* (1979) argued that on reduction of sulphur intake, more selenium would be used in place of sulphur and be incorporated into the rumen micro-

organisms leaving less selenium to be converted to unavailable forms. As microbial selenium is present in organic forms, Whanger *et al.* (1978) thought that it would be readily available to the animal. This would explain the increased selenium incorporation into wool and plasma (White and Somers, 1977; White, 1980) and its increased retention (Pope *et al.*, 1979) when sheep were on a sulphur deficient diet.

2.3.6 The Effects of Selenium on Rumen Metabolism

The addition of sulphur to ruminant diets, thereby increasing the amount of sulphur available to the microorganisms, has been shown to increase the amount of microbial protein in the rumen (Hume and Bird, 1970), stimulate bacterial activity (Bull and Vandersall, 1973), increase V.F.A. production (Whanger and Matrone, 1965) and alter the numbers (Gall, Thomas, Loosli and Huhtanen, 1951) and species (Whanger and Matrone, 1965) of the microbial population. In the light of the presented evidence concerning similarities in the metabolism of sulphur and selenium, one might expect selenium to affect the observations mentioned above.

2.3.6.1 Microbial Multiplication

Two series of experiments have revealed that selenium can have an effect on the microflora of the rumen. An *in vitro* study by Hidiroglou *et al.* (1968) revealed that rumen inoculum from selenium supplemented sheep had twice the total microbiological count and an altered species composition compared to inoculum from a selenium deficient sheep. Later

in vivo work by Hidiroglou and Lessard (1976) confirmed these results, and chain-forming cocci were the predominant organisms in rumen fluid from selenium supplemented sheep, while selemonads and bacteroides were the main groups in the rumen of unsupplemented sheep.

In culture trials Forsberg (1978) found subinhibitory and growth inhibitory selenium concentrations varied between the four bacterial species studied, which could account for the different species composition in the rumen fluid of the supplemented and unsupplemented sheep described above.

As selenium is incorporated into microbial proteins, and presumably influences bacterial species composition via this, an understanding of the effects of selenium on protein synthesis is necessary.

2.3.6.2 Protein Synthesis

After one of two groups of sheep on selenium deficient diets was dosed with selenium, Hidiroglou and Zarkadas (1976) added labelled methionine to the rumen of all sheep, and found that the group dosed with selenium incorporated more of the label into the rumen liquor, the bacterial fraction and the protozoa than the unsupplemented group. Analyses of bacterial protein showed that the levels of radioactivity of both cyst(e)ine and methionine were significantly higher for the selenium supplemented group. Their results suggested that the rate of methionine metabolism and its distribution in rumen bacterial proteins was dependent on the selenium status of the animal. As selenium had no effect on the amino acid composition of protein, the authors

thought there may have been increased rates of protein synthesis in the rumen of selenium supplemented sheep. This is an area for further investigation.

While selenium at low levels in the rumen might have stimulated protein synthesis, Khirwar and Arora (1976) could only show that synthesis was affected at high selenium concentrations. In an *in vitro* study using rumen liquor from sheep on selenium deficient diets they found that ^{35}S incorporation into bacterial protein was uniform at all levels of added selenite up to 1 mg Se/*l* of incubation mixture. At levels greater than this sulphur incorporation declined significantly as did microbial protein synthesis. The authors concluded that as selenium didn't affect the nitrogen to sulphur ratio in protein it must have affected the multiplication of microbes rather than being incorporated in place of sulphur. The decrease in microbial multiplication at high selenium concentrations was thought to be due to an increase in the ratio of selenoamino acids to the sulphur analogues, making microbial cell protein inactive.

Stadtman (1974), in a review introduction, explained that those enzyme systems which couldn't distinguish selenium from sulphur, incorporated selenium indiscriminantly for sulphur in many cellular constituents. Because of the greater reactivity and lower stability of seleno compounds, Stadtman thought the cell would encounter metabolic problems. In bacteria this would eventually lead to an inactivation of cell protein and eventual death of the organism.

2.3.6.3 Dry Matter Digestion

In 1958, Little, Cheng and Burroughs reported that selenium concentrations above 0.3 mg/*l* inhibited cellulose digestion by rumen microorganisms in an *in vitro* medium. At the same concentrations Billon, Edwards and Byrd (1975) found a decrease in dry matter disappearance during a 48 hour incubation. In a more extensive *in vitro* experiment, Martinez and Church (1970) found no change in % cellulose digested at selenium levels of 0.01 to 0.1mg/*l*. Levels of 1 to 5mg/*l* slightly reduced digestion while 7mg/*l* and above progressively depressed digestion.

These findings suggest that not only is the addition of selenium to the diets or rumen fluid not required for maximum cellulose digestion, but concentrations as low as 0.3mg/*l* can inhibit microbial cellulolytic activity. The mechanisms by which selenium causes these effects have not been described, but considering the described effects of selenium on bacterial species, it is possible that selenium concentrations of 0.3mg/*l* and above suppressed the growth of cellulolytic bacteria.

2.3.6.4 VFA Production

Following a weekly oral dose of 1mg sodium selenite to sheep on a purified diet, Hidiroglou and Lessard (1976) found no significant differences in the total VFA, acetic, propionic or butyric acid concentrations, in the rumen of sheep with or without the selenium supplement. The only VFA to be produced in greater amounts when sheep were supplemented with selenium was iso-valeric acid. This was thought to be a result of increased bacterial protein synthesis

(and presumably more protein degradation as iso-valeric acid is a product of protein metabolism). Analyses of the VFA molar % showed that the proportion of acetic acid, and the ratio of acetic to propionic acid, was higher in the selenium supplemented animals, while the proportions of propionic and butyric tended to be lower. These changes were probably due to the different rumen bacterial species observed between the two groups of sheep.

When sodium selenite doses were slightly greater (0.1mg/kg body weight) Farzaliev (1981) reported increased VFA concentrations and depressed rumen pH, and when added with animal fat caused a fall in acetic acid molar % and an increase in % propionic acid. Presumably the differences between the results of Hidiroglou and Lessard (1976) and Farzaliev (1981) are due to higher selenium supplementations in the latter's experiments, although it is difficult to draw conclusions because experimental methods were not detailed when Farzaliev's work was published in abstract form.

As alterations in microbial populations and amounts of cellulose digested are reflected in the amounts and composition of VFA produced (Warner, 1964), the established effects of high levels of selenium on the former factors should also influence VFA production. In an *in vitro* incubation Billon, Edwards and Byrd (1975) found that as the levels of inorganic selenium increased from 0.3 to 3mg/*l* acetic, propionic and butyric acid concentrations decreased concommitant with an increased pH. High selenite levels were also inhibitory to the microbial fermentative activity measured as gas production according to Forsberg (1978). As VFA production occurs

concurrently with methane release, and some VFA may be precursors of methane (Isaacson, Hinds and Bryant, 1975), the depressing effect of selenium on fermentation would also be associated with the decreased VFA production described by Billon *et al.* (1975) above.

2.3.7 Summary

Since the first investigation of the effects of selenium in the rumen almost twenty years ago, it has been conclusively established that it can be incorporated into bacterial proteins as selenoamino acids. It appears that selenium may be competitive with sulphur in the rumen, the two being degraded to selenite and sulphide respectively, by similar enzymic pathways. While the incorporation pathways of sulphur into amino acids and proteins have been documented, corresponding selenium pathways remain unknown.

Although selenium has been shown to be essential for enzyme activity in some anaerobic bacteria, there is no evidence that it is essential for rumen bacterial activity, however there are some reports that it may stimulate bacterial metabolism. While selenium has not been shown to increase carbohydrate digestion, there is circumstantial evidence that it may increase protein synthesis (Hidiroglou and Zarkadas, 1976), and a recent report that it can increase VFA production (Farzaliev, 1981). However other authors have reported that low levels of selenium have no effect on these bacterial functions. At concentrations of 0.3mgSe/*l* and above, the major bacterial activities, VFA production, digestion of dry matter, and protein synthesis, decline.

CHAPTER 3

METHODS

3.1 ARTIFICAL RUMEN INCUBATIONS

3.1.1 Collection of Rumen Fluid

Rumen liquor was collected from a fistulated poll dorset ram, penned indoors and fed 500g of good quality lucerne hay per day. The animal had not been dosed with selenium in the previous six months. Prior to collection of rumen fluid the animal was semi-starved over the weekend period, receiving only 50g of hay each day but given free access to drinking water.

Before the sheep was fed on Monday morning two to three litres of fluid were removed through the rumen fistula, strained through four layers of muslin cloth into a pre-warmed thermos flask, and then transported to the laboratory.

In order to cause the least amount of disturbance to the microbial population the above procedure was carried out as quickly as possible (within 15 minutes). Also every effort was made to keep the fluid in anaerobic conditions, although Hobson (1971) found that the anaerobic status of rumen fluid standing in air for short periods was similar to fluid *in vivo*.

3.1.2 Addition of Selenium to Incubation Inoculum

In all studies sodium selenate was added to the incubation flasks prior to addition of the incubation medium.

By pipetting small volumes of stock solutions of $NaSeO_4$ into the flasks (and making the volume to 1ml with distilled water), the selenium concentrations following addition of incubation medium ranged from 0 to 80mg/*l* depending on the particular experiment. Four or five replicates were used at each selenium concentration.

3.1.3 Hay Substrate Incubations

For all digestion experiments where hay was used as the substrate, the digestion was based on the first stage of the widely used *in vitro* digestibility method of Tilley and Terry (1963).

An artificial saliva developed by Baumgardt, Cason and Taylor (1962) was used to buffer and nourish the bacteria. In addition to the standard McDougall buffer (McDougall, 1948) (table 1) (without $MgCl_2$ as Mg was added as $MgSO_4$) the artificial saliva contained the substrates sulphate, glucose and urea for growth. The artificial saliva was heated to 38° then mixed with the strained rumen fluid in a 4:1 ratio to provide the incubation mixture detailed in Table 2. The temperature of the mixture was kept at 38° by standing its dispenser in a waterbath. The pH was maintained at 6.9 and CO_2 gas was bubbled continuously through the incubation mixture to maintain anaerobic conditions.

Table 1 - Composition of McDougall's Buffer

Component	Concentration (g/l)
$NaHCO_3$	9.80
Na_2HPO_4 (anhydrous)	3.71
KCl	0.57
NaCl	0.47
$MgCl_2$ (anhydrous)	0.06
$CaCl_2$ (anhydrous)	0.04

Table 2 - Composition of Incubation Mixture for Use With a Hay Substrate

Component	Concentration
Rumen liquor	200ml/l
McDougall's buffer	800ml/l
Urea	730mg/l
Glucose	730mg/l
$MgSO_4.7H_2O$	96mg/l

50ml of the incubation mixture were dispensed into 125ml conical flasks containing 500mg of lucerne hay (that had been ground through a 1mm screen) and a known amount of previously added sodium selenate. The flasks were continuously flushed with CO_2 during dispensing of the incubation mixture and stoppered immediately with a rubber bung fitted with a Bunsen valve (Bently, Johnson, Vanecko and Hunt, 1954) allowing release of fermentation gases but preventing air from entering.

The flasks were placed in an oven at 38° for the duration of the incubation and hand swirled three times daily to simulate ruminal mixing.

3.1.4 Starch Substrate Incubations

The method was based on that used successfully by Khirwar and Arora (1976) in similar experiments examining the effects of selenium on protein synthesis. The main difference between this and the artificial rumen digestion with hay substrate, was the composition of the artificial saliva. Additional sulphur (as Na_2SO_4) was added to maintain a 10:1 N:S ratio which Moir (1970) and Khirwar and Arora (1976) showed to be optimal for bacterial growth in this type of trial. The amount of sulphur added (53mgS/*l*) was also above the minimal addition (43mgS/*l*) required for protein synthesis from non-protein nitrogen in long term incubations (Durand and Kawashima, 1980).

As no hay substrate was added to the flasks, starch was dissolved in the artifical saliva. Before the SRL was added to the artificial saliva it was centrifuged at 750 x g for 5 minutes at 38°C to remove feedstuff debris, protozoa and the heavier bacteria. The composition of the incubation mixture is detailed in Table 3.

Table 3 - Composition of Incubation Mixture Containing Starch

Component	Concentration
McDougall's buffer	333ml/*l*
Rumen fluid	333ml/*l*
Water	333ml/*l*
Urea	1.17g/*l*
Starch (soluble)	16.7g/*l*
Na_2SO_4	233mg/*l*

30ml of incubation mixture was added to each flask, the latter containing a known amount of added sodium selenate. After 24 hours of incubation the pH of each flask was measured. In all other respects the procedure was the same as that used in the incubations with hay substrate described previously.

3.1.5 Length of Incubation Period

An initial experiment was undertaken to determine the optimum incubation time to measure any effects of Se on VFA production with hay used as a substrate as Billon, Edwards and Byrd (1975) demonstrated that the effects varied with incubation time.

It was found that incubating all flasks for 48 hours and sampling periodically was unsuitable as the removal of a significant volume of inoculant at each sampling, and the associated disturbance to the microbial population, gave unreliable results. This occurred probably because the removal affected the remaining microorganisms and their metabolism (Warner, 1966). Therefore four replicates at each of five selenium levels were incubated for periods of 3, 6, 10.5, 21, 28.5 or 48 hours giving a total of 120 flasks.

Billon, Edwards and Byrd (1975) observed effects of selenate on the dry matter disappearance of hay only after 48 hours of incubation. Therefore, to measure the change in dry matter with added selenate it was decided to adopt the 48 hour incubation period. This incubation time was also used by Tilley and Terry (1963) for forage digestibility

studies.

For all other incubations, the more common 24 hour period (e.g. Hidiroglou, Heaney and Jenkins, 1968; Hidiroglou, Jenkins and Knipfel, 1974; Khirwar and Arora, 1976) was used.

3.2 MEASUREMENT OF PROTEIN CONCENTRATION

3.2.1 Protein Precipitation

The method of precipitating rumen bacterial protein with trichloroacetic acid (TCA) has been widely used following *in vitro* incubations (Cline, Hershberger and Bently, 1958; Hidiroglou, Heaney and Jenkins, 1968; Paulson, Baumann and Pope, 1968). However Shultz and Shultz (1970) have stated that this method "does not precipitate microbial nitrogen as completely as does the tungstic acid procedure". Therefore the tungstic acid method (Winter, Johnson and Dehority, 1964) was used in this experiment.

Following incubation, 4ml of incubation mixture were removed for VFA analyses and 26ml transfered to 50ml centrifuge tubes. 6.5ml of 0.535M sulphuric acid and 6.5ml 10% sodium tungstate were added to the centrifuge tubes and then vortexed. After standing overnight at 4^{o} the mixture was centrifuged at 2000 x g for 20 minutes at 4^{o}.

The supernatant was discarded and the pellet resuspended in 10ml distilled water. 2.5ml of sulphuric acid and 2.5ml of Na tungstate solution were added and the solution mixed. After standing a minimum of 4 hours, the mixture was again centrifuged at 2000 x g for 20 minutes. After the procedure

described immediately above was repeated, the supernatant was discarded and the protein containing pellet was dissolved in 30ml 0.1M NaOH ready for analyses. (For an alternative method of centrifugation see appendix 1.)

3.2.2 Protein Assay

The method of Lowry, Rosenbrough, Farr and Randall (1951), shown by Khirwar and Arora (1976) to satisfactorily determine protein content of tungstic acid precipitated microbial protein, was used in these determinations. Bovine serum albumin (Sigma) was used as a reference standard. This method was also compared to the more recent and convenient Coomassie Brilliant Blue assay for proteins (Bradford, 1976), but the latter proved to be unsuitable (see appendix 2).

3.3 VOLATILE FATTY ACID ANALYSES

3.3.1 Preparation and Storage of Samples

After incubation of the artificial saliva and rumen fluid mixture, a 4.0ml sample was taken and 1.0ml of a combined internal standard (crotonic acid, 250mg/100ml) and deproteinising solution (20% metaphosphoric acid v/v) was added.

The samples were then centrifuged at 2000 x g for 10 minutes to precipitate protein and any residual hay if present. The supernatant was decanted off and stored at -18^{o}. At low pH and temperature fermentation ceases and the samples could be stored for long periods prior to analyses.

3.3.2 Equipment Used for VFA Analyses

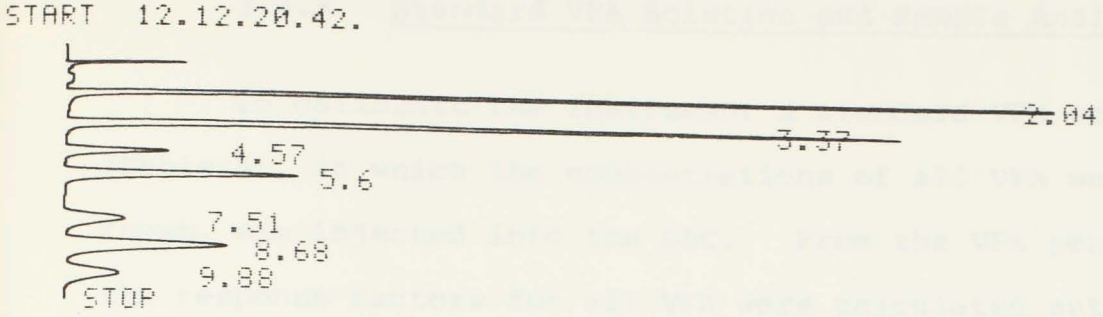
A Varian Aerograph Series 2800 chromatograph, fitted with a flame ionisation detector, had a formic vapour trap (described by Pileire (1978)) introduced into the carrier gas line just prior to the injection port. Carrier gas was passed through 95% formic acid vapour which increased the reproducibility of VFA analyses (Joe, 1982), mainly by decreasing the tailing of VFA peaks enabling a more accurate measurement of peak areas.

The machine was fitted with a 2400mm glass column of 3mm internal diameter. The column was cleaned in chromic acid then soaked in 85% phosphoric acid overnight before drying. The column was packed with Tenax GC coated with 6% FAL-M (Shimadzu Corporation, Kyoto) and plugged at both ends by glass wool pretreated with 85% phosphoric acid. The phosphoric acid decreased peak tailing of the VFA by eliminating the polar sites on the column (Pileire, 1978). After packing the column it was conditioned for two days at 165°. The GLC parameters were then set up as detailed in table 4 ready for VFA analyses. Nitrogen was used as the carrier gas and a hydrogen/dry air mixture for the flame.

Table 4 - GLC Parameters for VFA Analyses

Column temperature	135°
Injection port temperature	145°
Detector temperature	150°
Nitrogen flow	40ml/min
Air flow	400ml/min
Hydrogen flow	40ml/min

Figure 3a: Data recorder output of a VFA standard

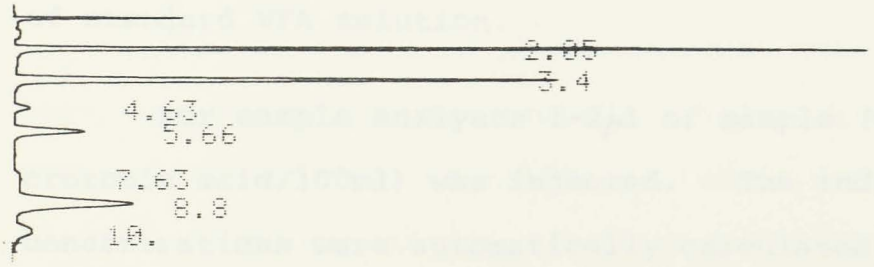


	#	NAME	TIME	WINDOW	F1/F2	C1/C2
0	1	CROT	8.66	5	1	50
0	2	ACET	2		1.95	250
0	3	PROP	3.35		1.262	125
0	4	I-BUT	4.56		1.03	20
0	5	BUT	5.9		1.04	50
0	5	BUT	5.6		1.04	50
0	6	I-VAL	7.5		0.9029	20
0	77	LMT-D				
0	7	VAL	9.8		0.9249	20

END

#	NAME	TIME	CONC	MK	AREA
2	ACET	2.04	250.1466		330580
3	PROP	3.37	124.864		277053
4	I-BUT	4.57	20.1653		54603
5	BUT	5.6	49.4536		134308
6	I-VAL	7.51	20.1341		61488
1	CROT	8.68		V	145286
7	VAL	9.88	20.2992	V	64078
	TOTAL		485.0629		1067400

Figure 3b: Data recorder output of a sample



C-R1A
SMPL # 00
FILE # 1
REPT # 233
METHOD 43

#	NAME	TIME	CONC	AREA
2	ACET	2.05	174.4661	182803
3	PROP	3.4	106.1856	186802
4	I-BUT	4.63	4.3001	9232
5	BUT	5.66	19.8187	42674
6	I-VAL	7.63	3.4865	8442
1	CROT	8.8		115190
7	VAL	10.	9.468	23696
	TOTAL		317.7252	568840

3.3.3 Standard VFA Solution and Sample Analyses

To calibrate the instrument a standard VFA solution (Table 5), in which the concentrations of all VFA were known, was injected into the GLC. From the VFA peak areas the response factors for all VFA were calculated automatically by a Shimadzu Chromatopac C-RIA, data processor (Shimadzu Corporation, Kyoto).

Table 5 - Composition of Standard VFA Solution

Acid	Concentration (mg/100ml)
Acetic	250
Propionic	125
Iso-Butyric	20
Butyric	50
Iso-Valeric	20
Crotonic	50
Valeric	20

Figure 3a shows the trace and output following a 1μl injection of standard VFA solution.

For sample analyses 1-2μl of sample (containing 50 mg crotonic acid/100ml) was injected. The individual VFA concentrations were automatically calculated from the peak areas and response factor - an example of the output of a sample analysis is shown in figure 3b.

3.4 MEASUREMENT OF THE CHANGE IN DRY MATTER

Following a 48 hour incubation period with hay substrate, the contents of each flask were filtered through a weighed gooch crucible (porosity 1) and washed several times with hot distilled water. The crucible containing the residue was then dried overnight at 105^{o} , cooled in a desiccator and weighed to determine the weight of undigested residue. To determine dry matter not arising from the hay substrate, a blank, containing no hay, was included in the incubations.

The following formula was used to determine the % of dry matter digested:

$$\% \text{ hay digested} = \frac{\text{Hay D.W.} - (\text{Residue D.W.} - \text{Blank D.W.}) \times 100}{\text{Hay D.W.}}$$

D.W. = dry weight

3.5 SELENIUM DETERMINATION

The two stages of determination, digestion and measurement, are based upon Watkinson's original 1966 method. Following a modified digestion procedure (Watkinson, 1979) Se was measured by the semi-automated fluorimetric method of Brown and Watkinson (1979).

The amount of Se in rumen fluid, incubation medium and hay could be measured down to a level of 400 picograms with an error of 1%.

CHAPTER 4

RESULTS

4.1 THE SELENIUM CONCENTRATION OF SRL USED FOR *IN VITRO* INCUBATIONS

The concentration of selenium in SRL (0.006mg/*l*) represented a five fold dilution of the concentration in the diet (0.03mgSe/kg). In the absence of published data on the selenium content of sheep's rumen fluid, it is unknown whether this is a representative level. Khirwar and Arora (1976) found the concentration in SRF was 0.28mg Se/*l* when the animal's diet contained 0.08 mg Se/kg hay. However as their SRF was obtained from a cow fed *ad lib.*, and previous selenium dosing of the animal was not mentioned, a comparison between results may not be valid, because of a different selenium status of their animals.

The selenium concentration of the incubation mixture containing starch substrate was 0.009 mg/*l*, and of the hay used in the hay substrate mixtures, 0.06 mg/kg.

4.2 THE EFFECT OF SELENATE ON THE DIGESTION OF A STANDARD HAY SUBSTRATE BY MICROORGANISMS *IN VITRO*

The proportion of hay digested in flasks containing no selenate was $64.4 \pm 1.6\%$ which compares favourably with the average figure of 65.5% calculated from three other digestibility experiments, and the *in vivo* digestibility value of 63.3%.

Figure 5 represents the data in appendix 3 and shows the effect of selenate concentrations from 0 to 5 mg/*l* on the % hay digested over 48h. Half the selenate levels were below 0.5mg/*l* as it was hoped to show an effect of selenate at low concentrations. A significant decline in % dry matter digested occurred from 0 to 0.3mg Se/*l* but the subsequent rise at 0.4mg Se/*l* was not significant. Above 0.4mg Se/*l* the amount of hay digested declined fairly linearly to 52.5% at 5mg Se/*l*.

4.3 CHANGES IN PROTEIN CONCENTRATIONS WITH ADDED SELENATE

The pre-incubation levels of protein were 149 and 114 mg/*l* for starch and hay substrate incubation mixtures respectively. The protein concentration was higher in the starch substrate mixture because it contained a greater proportion of rumen fluid. Strained rumen liquor (SRL) contained 572 ± 12 mg protein/*l* and the centrifuged rumen liquor used for the starch substrate mixture, 452 ± 10 mg/*l*.

After 24h incubation the protein concentration of the hay substrate mixture had increased from 114 ± 9 to 121 ± 18 mg/*l*, and in the starch substrate mixture from 149 to 306 mg/*l*.

The data from which the graphs and table showing the effects of selenate on protein synthesis are derived, are detailed in appendices 4 and 5.

Although table 6 shows selenate concentrations up to 3mg/*l* slightly elevated the net amount of protein synthesised in the hay substrate incubations, the differences were found

FIGURE 4: The effect of high levels of selenium on the synthesis of microbial protein during a 24 hour incubation using hay as a substrate.

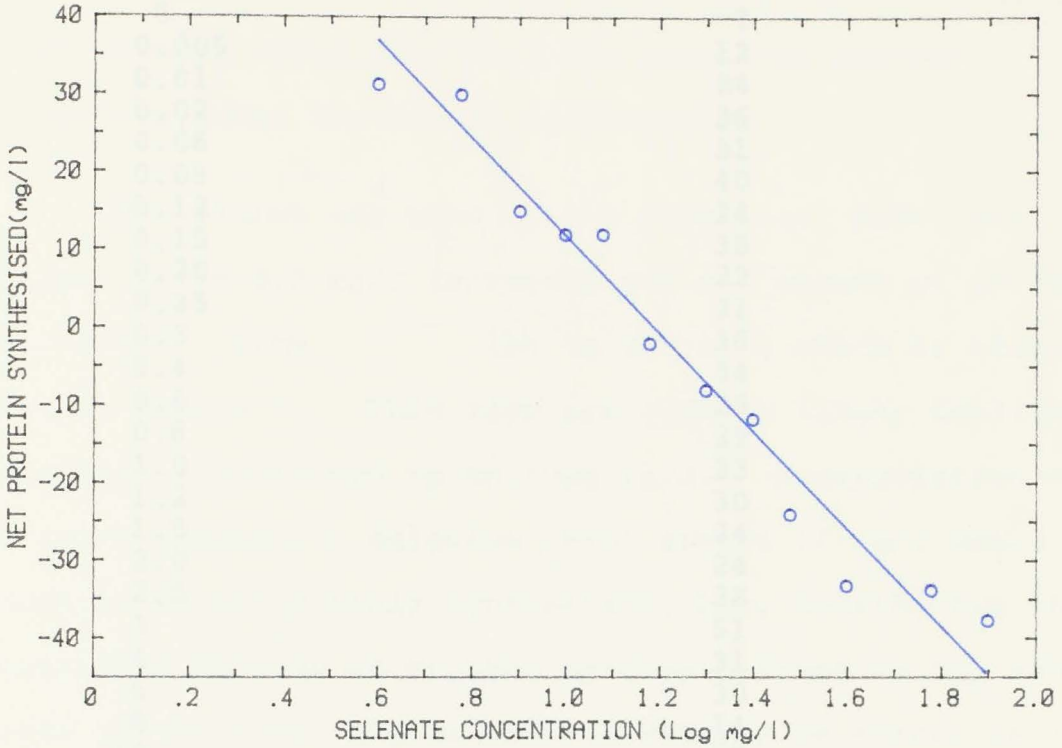


Figure 5: The effect of selenium on the amount of standard hay substrate digested.

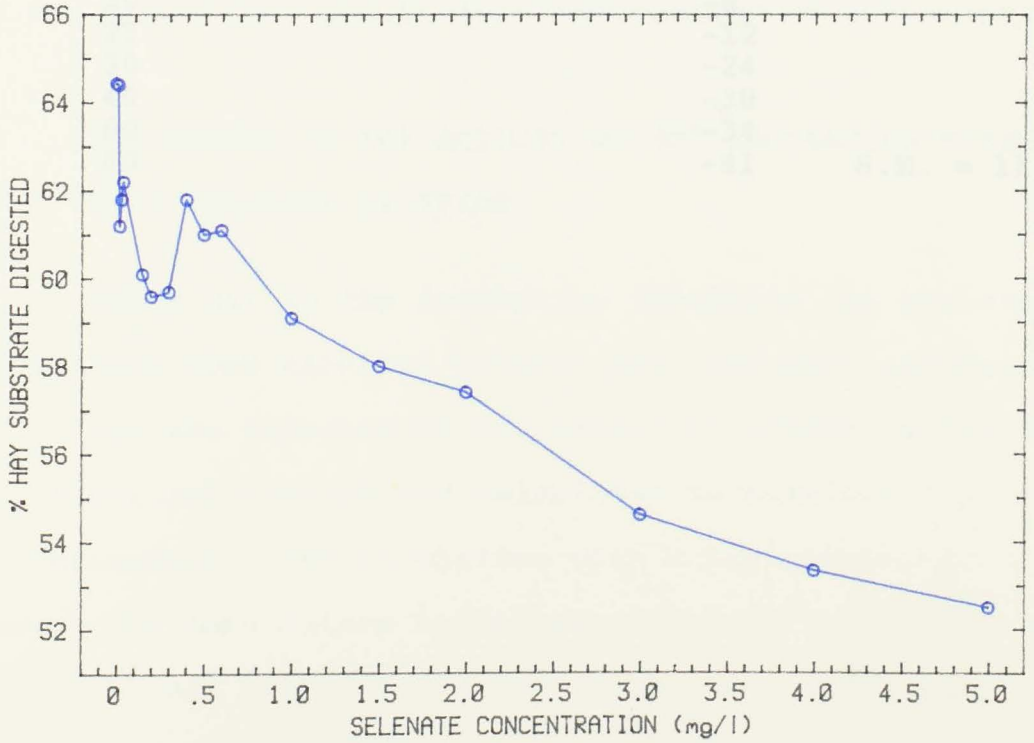


Table 6: THE EFFECT OF SELENIUM ON PROTEIN SYNTHESIS DURING A 24 HOUR INCUBATION WITH A HAY SUBSTRATE

Selenate concentration (mg/l)	Net protein synthesised (mg/l)	
0	7	
0.005	12	
0.01	24	
0.02	36	
0.06	31	
0.08	40	
0.12	24	
0.15	30	
0.20	22	
0.25	31	
0.3	36	
0.4	34	
0.6	25	
0.8	39	
1.0	23	
1.2	30	
1.5	24	
2.0	24	
2.5	38	
3	51	
4	31	
6	30	
8	14	
10	12	
12	11	
15	-2	
20	-8	
25	-12	
30	-24	
40	-38	
60	-34	
80	-41	S.E. = 11.9

to be not significant.

Selenate concentrations above 3mg/*l* resulted in a decrease in protein synthesis as figure 4 indicates. Protein levels declined logarithmically up to 80mg Se/*l*, and at concentrations above 15mg Se/*l* the amount of protein degraded exceeded the amount synthesised.

When starch was used as the substrate, additions of selenate up to 0.3 mg/*l* increased the net amount of protein synthesised from 158 to 255 mg/*l* which is clearly shown in figure 6. This rise preceeded a linear decline in protein synthesised up to 10mg Se/*l*. Extrapolation of the curve suggests a selenium level around 13 mg/*l* would result in no net protein synthesised, but, considering the logarithmic decline in protein synthesis found in hay substrate incubations, the selenium level may be nearer to the value of 15mg Se/*l* found in hay substrate incubations, if the curve flattens off at the higher selenium concentrations.

4.4 THE CHANGE IN THE ACIDITY OF THE INCUBATION MIXTURE WITH SELENATE ADDITION

Using hay as the incubation substrate, no noticeable differences were recorded between pre- and post incubation pH. This was expected as the amount of buffer included in the incubation mixture was calculated to maintain a pH of 6.9 throughout a 48h incubation with a hay substrate. However the more dilute buffer, adopted from the experiments of Khirwar and Arora (1976), for incubations with starch as a substrate, was insufficient to maintain a pH of 6.9, and

FIGURE 6: The effect of selenium on protein synthesis during a 24 hour incubation using starch as the carbohydrate substrate.

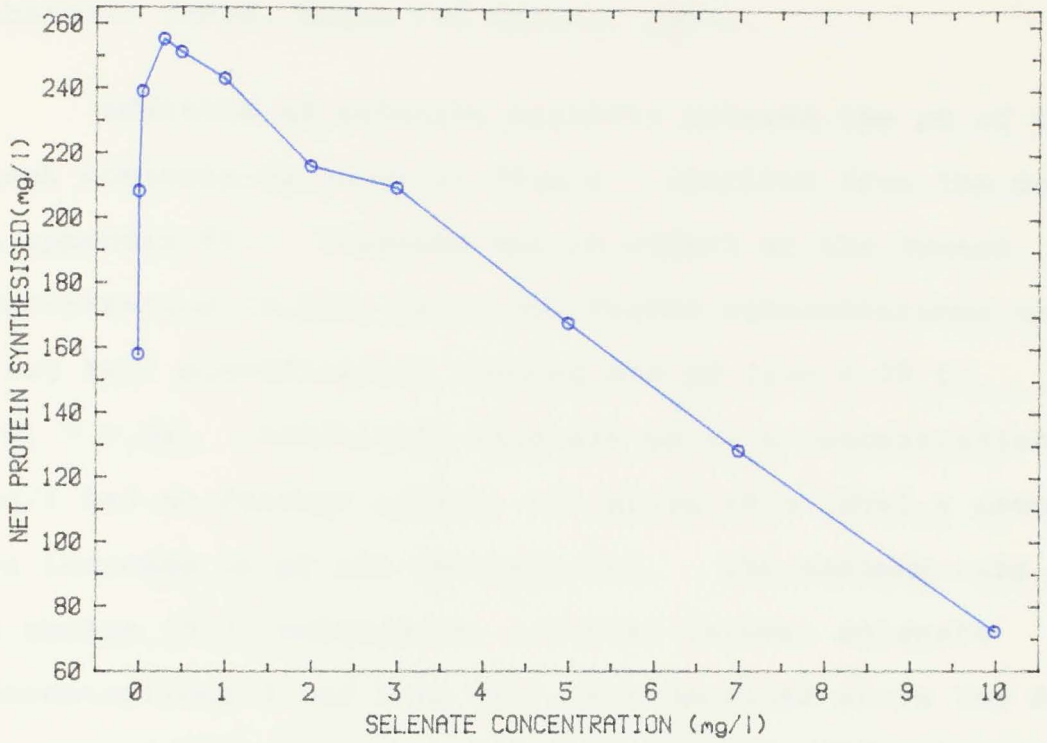
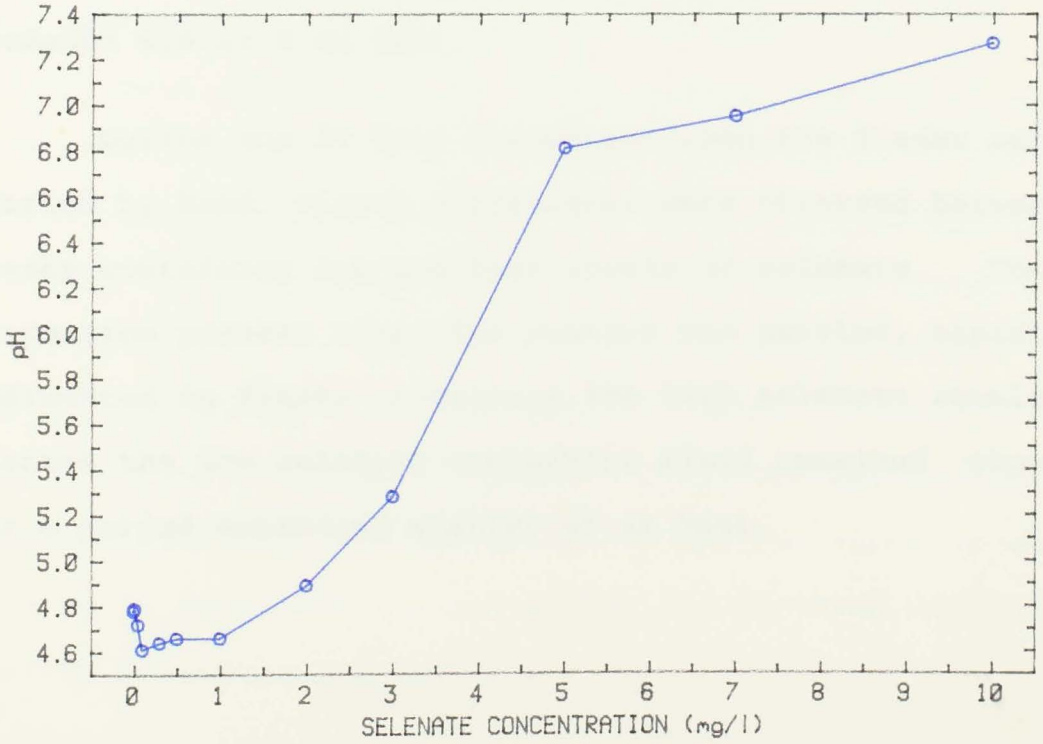


FIGURE 7: The effect of selenium on the acidity of the incubation mixture with a starch substrate.



after 24h of incubation the pH had dropped to below 4.8 in flasks with no added selenate. The decrease in pH was probably a result of an accumulation of lactic acid in the medium, usual when starch is used as the carbohydrate substrate (Reid, Hogan and Briggs, 1957).

Addition of selenate markedly altered the pH of the flask contents as shown in figure 7 (derived from the data in appendix 6). Selenate had no effect at the lowest concentration (0.01mg Se/*l*) but higher concentrations up to 0.1mg Se/*l* significantly reduced the pH from 4.78 to 4.61 ± 0.02. Additional selenate up to a concentration of 1mg/*l* had no further effect, but above this level a progressive increase in pH was demonstrated. The maximum rate of pH change (0.77 units/mgSe) occurred between selenate concentrations 3 and 5 mg Se/*l*, but declined above 5mg Se/*l* to 0.15 pH units/mg Se. This was probably because of the equilibrating effects of the buffer medium when the pH exceeded 6.8 at 5 mg Se/*l*.

During the 24 hour incubation when the flasks were swirled by hand, visual differences were observed between flasks containing low and high levels of selenate. The cloudy suspension present after the mixture was swirled, rapidly sedimented in flasks containing the high selenate levels, whereas the low selenate containing fluid remained cloudy for a period exceeding quarter of an hour.

4.5 THE INFLUENCE OF SELENATE ON THE PRODUCTION OF VFA

4.5.1 Starch Substrate Incubations

The data presented in appendices 7 and 8, have been used for all tables and graphs in this section.

Selenate additions significantly affected the amounts of all VFA produced except iso-valeric and valeric acid as shown in Table 7. The absence of any significant differences in these higher VFA was probably due to the large variability (0.68 and 0.90 for iso-valeric and valeric S.E. respectively) associated with the determination of the low amounts of these acids. In several samples the concentrations were so low that the data recorder was unable to register their chromatograph peaks on elution.

Figure 8 shows that with increasing concentrations of selenate, the amounts of VFA produced during incubation also increased initially, acetic, propionic and butyric acid concentrations reaching their maximum at selenate levels of 0.3, 2.0, and 0.5mg/*l* respectively. Above these selenate concentrations, the VFA production declined slightly to 3 mg Se/*l*, then steeply and fairly linearly up to 10 mg Se/*l*. At the latter selenate concentration, the VFA concentrations were approximately equal to their concentrations with no selenate added. Therefore any addition of selenate such that the concentration did not equal or exceed 10mg Se/*l*, increased the production of the three major VFA, acetic, propionic and butyric.

The difference in the response of individual acids to

Table 7 : THE EFFECT OF SELENIUM ON THE AMOUNTS OF VFA PRODUCED DURING INCUBATION WITH STARCH SUBSTRATE

Selenate concentration (mg/l)	Concentrations of VFA (mg/100ml)						
	Acet.	Prop.	I-But.	But.	I-Val.	Val.	TVFA
0	37.8	22.6	5.7	2.0	1.1	0.9	70
0.01	42.1	22.6	5.1	1.3	-	-	71
0.05	59.6	33.6	4.2	2.9	0.2	0.6	101
0.1	64.8	39.1	4.5	5.2	0	0.23	114
0.3	82.8	50.8	7.3	8.3	1.45	4.0	152
0.5	73.8	49.1	9.2	11.7	1.38	0.8	146
1.0	70.7	52.9	5.5	9.2	2.35	4.3	144
2.0	68.3	58.3	2.3	9.5	-	-	138
3.0	73.6	54.6	2.0	10.3	2.3	.05	141
5.0	64.6	36.1	1.0	6.0	0.5	1.0	109
7.0	55.6	30.8	1.0	5.6	1.3	1.5	96
10.0	40.6	19.6	1.2	1.2	1.3	1.2	65
S.E.	4.5	4.4	0.7	2.0	0.68	0.90	8.9
Statistical Significance	**	**	**	**	N.S.	N.S.	**

Table 8 : THE EFFECT OF SELENIUM ON THE PROPORTIONS OF VFA PRODUCED DURING INCUBATION WITH A STARCH SUBSTRATE

Selenate concentration (mg/l)	VFA molar %						Acet/Prop ratio
	Acet.	Prop.	I-But.	But.	I-Val.	Val.	
0	54.8	32.2	8.0	2.6	1.9	0.9	1.71
0.01	59.2	31.8	7.2	1.8	-	-	1.88
0.05	58.9	33.1	4.3	2.7	0.2	0.8	1.78
0.1	57.0	34.3	3.9	4.6	0	0.2	1.67
0.3	54.7	33.5	4.8	5.4	1.1	0.6	1.66
0.5	50.7	33.4	6.3	8.2	0.9	0.6	1.56
1.0	49.6	36.1	3.8	6.5	2.2	1.7	1.40
2.0	49.3	42.1	1.7	7.0	-	-	1.17
3.0	52.6	38.6	1.4	7.3	0.1	0	1.39
5.0	59.2	33.0	0.9	5.4	0.5	0.9	1.80
7.0	58.3	32.1	1.0	5.7	1.4	1.6	1.82
10.0	62.7	30.0	1.8	1.7	1.9	1.8	2.10
S.E.	1.76	1.58	0.52	0.74	0.56	0.49	0.12
Statistical Significance	**	**	**	**	N.S.	N.S.	**

Acet. = acetic; Prop. = propionic; I-But. = Iso-Butyric; But. = Butyric; I-Val = Iso-Valeric; Val. = Valeric; TVFA = Total VFA

'**' denotes significance at 1% level (using analysis of variance)
N.S. denotes not significant.

FIGURE 8: The effect of selenium on the net production of VFA during incubation with a starch substrate.

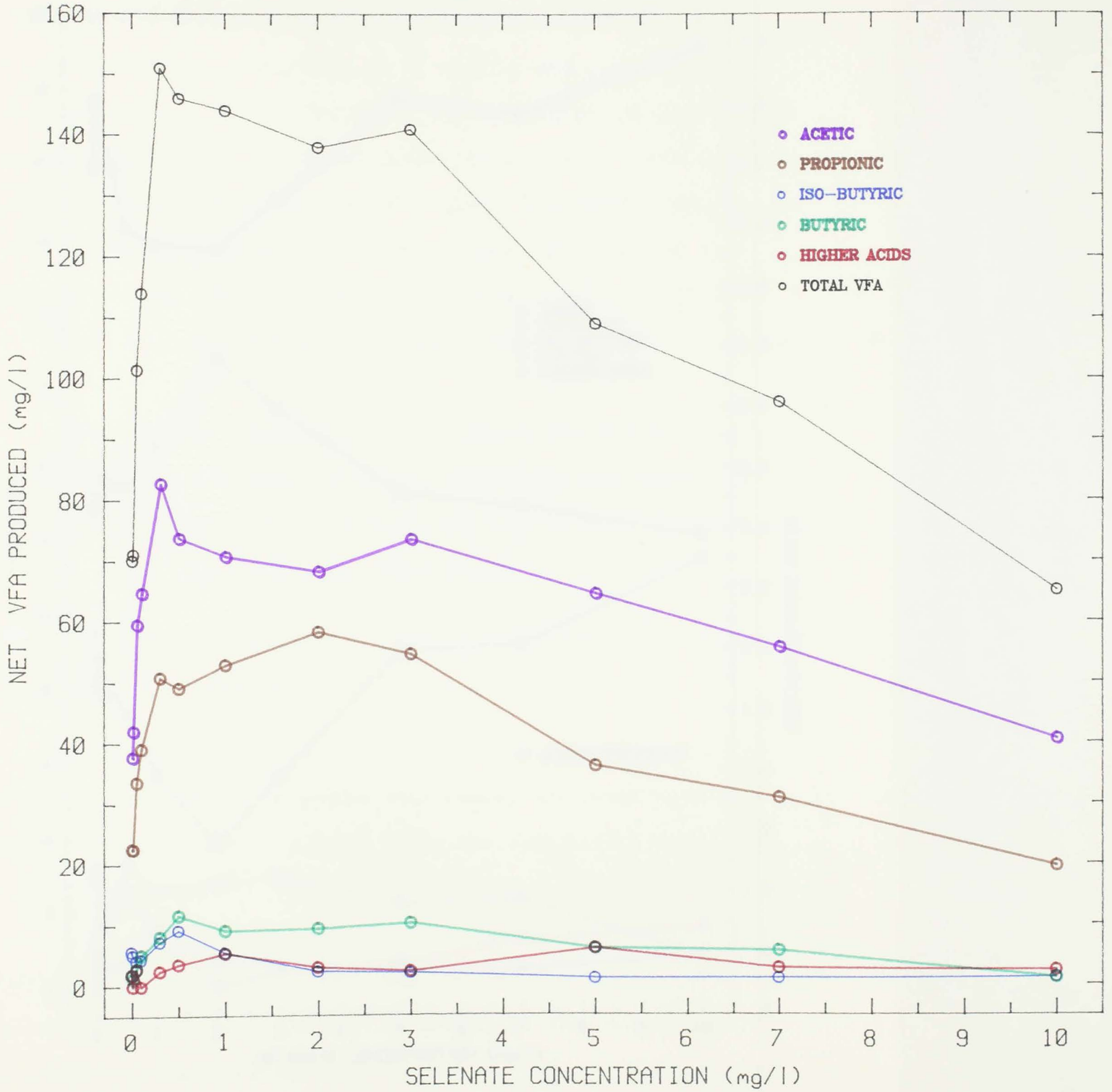
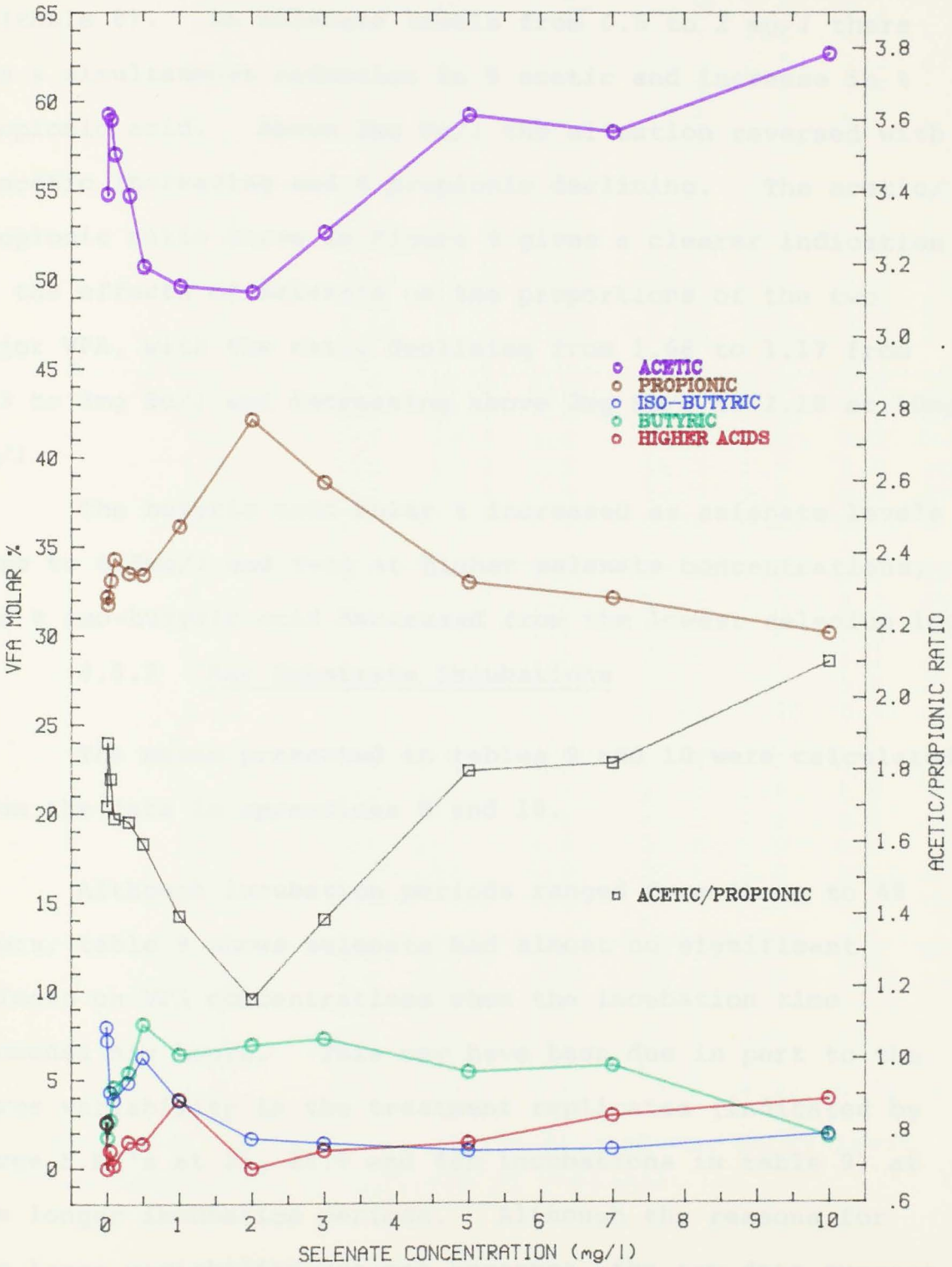


FIGURE 9 :The effect of selenium on the proportions of VFA produced during a 24 hour incubation with a starch substrate.



selenate, was reflected in changes in the VFA proportions or molar %. Although the production of acetic, propionic and butyric acids increased up to selenate concentrations of 0.3mg/*l*, no significant changes occurred in their molar % (table 8). At selenate levels from 0.5 to 2 mg/*l* there was a simultaneous reduction in % acetic and increase in % propionic acid. Above 2mg Se/*l* the situation reversed with % acetic increasing and % propionic declining. The acetic/propionic ratio curve in Figure 9 gives a clearer indication of the effects of selenate on the proportions of the two major VFA, with the ratio declining from 1.66 to 1.17 from 0.3 to 2mg Se/*l* and increasing above 2mg Se/*l* to 2.10 at 10mg Se/*l*.

The butyric acid molar % increased as selenate levels rose to 0.5mg/*l* and fell at higher selenate concentrations, and % iso-butyric acid decreased from the lowest selenium level.

4.5.2 Hay Substrate Incubations

The means presented in tables 9 and 10 were calculated from the data in appendices 9 and 10.

Although incubation periods ranged from three to 48 hours, table 9 shows selenate had almost no significant effects on VFA concentrations when the incubation time exceeded six hours. This may have been due in part to the large variability in the treatment replicates (indicated by large S.E.'s at 21, 28.5 and 48h incubations in table 9) at the longer incubation periods. Although the reasons for the large variability are not apparent, the raw data suggest that it may be a result of selenate addition, because the S.E. of groups containing no selenate does not increase

Table 9: THE EFFECT OF SELENIUM ON VFA CONCENTRATIONS AT DIFFERENT INCUBATION PERIODS, WITH A HAY SUBSTRATE

Incubation Period (h)	Selenate Concentration (mg/l)	VFA Concentration (mg/100ml) Acet.	Prop.	I-But.	But.	I-Val.	Val.	TVFA
0	0	48	32	0	7	1.0	0	88
3	0	68.6	44.2	2.20	10.9	1.3	2.1	129
	0.02	70.7	45.1	2.23	11.1	1.6	2.7	134
	0.2	69.8	44.2	2.23	11.0	1.7	2.3	131
	1.0	68.6	44.3	2.17	10.8	1.4	2.3	130
	5.0	63.4	42.6	2.00	8.4	1.2	1.6	121
	S.E.	1.35	0.94	0.05	0.87	0.19	0.20	2.2
		*	*	*			*	*
6	0	83.9	56.4	2.3	13.8	1.8	4.3	163
	0.02	95.6	63.2	2.5	15.9	1.3	4.0	179
	0.2	88.6	60.3	2.3	13.8	1.5	3.8	170
	1.0	84.6	58.0	2.2	12.9	1.6	4.2	163
	5.0	86.1	60.8	2.3	12.4	1.6	3.5	167
	S.E.	2.00	1.29	0.08	0.36	0.23	0.13	3.8
		**	*		**		*	
10.5	0	110	66.6	2.5	16.6	1.9	7.3	205
	0.02	116	69.1	3.4	16.7	1.8	7.3	214
	0.2	114	68.4	2.4	16.5	1.6	7.2	209
	1.0	112	68.0	2.5	16.3	1.7	7.6	208
	5.0	108	67.9	2.4	16.7	1.9	6.9	204
	S.E.	2.4	1.38	0.42	0.32	0.19	0.26	3.9
21	0	156	93.6	2.7	22.4	1.6	8.2	285
	0.02	162	95.5	3.0	22.3	1.3	8.2	292
	0.2	170	102.8	3.0	21.6	1.2	8.6	310
	1.0	173	103.5	2.8	20.6	1.2	8.2	309
	5.0	144	89.5	3.2	15.6	0	9.0	263
	S.E.	8.2	4.6	0.23	1.28	0.25	0.49	15.5
					*			
28.5	0	192	108	3.3	25.9	2.0	8.6	339
	0.02	192	111	3.5	26.3	2.5	8.8	342
	0.2	176	103	3.4	21.1	1.3	8.4	312
	1.0	186	112	3.4	23.0	1.6	8.9	334
	5.0	183	107	4.0	35.1	3.4	10.9	342
	S.E.	8.9	4.6	0.28	3.8	0.65	0.47	16.4
							*	
48	0	212	112	4.4	21.0	3.3	9.8	362
	0.02	214	123	4.8	28.0	3.8	10.5	385
	0.2	217	129	4.8	27.1	3.9	10.0	391
	1.0	209	123	4.1	24.1	3.2	10.2	373
	5.0	232	139	4.2	35.3	3.4	11.5	426
	S.E.	16.4	9.3	0.25	4.1	0.27	0.56	30.0

'*' = significant at 5% level; '**' = significant at 1% level

Differences determined by analysis of variance.

Table 10: THE EFFECT OF SELENIUM ON THE PROPORTIONS OF VFA PRODUCED AT DIFFERENT INCUBATION PERIODS, USING A HAY SUBSTRATE

Incubation Period (h)	Selenate Concentration (mg/l)	VFA molar %						Acet./ Prop. Ratio
		Acet.	Prop.	I-But.	But.	I-Val.	Val.	
0		54.5	36.4		7.9	1.1	0	1.5
3	0	53.0	34.2	1.70	8.4	1.0	1.6	1.55
	0.02	52.9	33.8	1.67	8.3	1.2	2.0	1.57
	0.2	53.3	33.7	1.70	8.4	1.3	1.8	1.58
	1.0	52.9	34.2	1.67	8.3	1.1	1.8	1.55
	5.0	52.5	35.3	1.66	7.0	1.0	1.3	1.49
	S.E.	0.25	0.18 **	0.03	0.70	0.16	0.17	0.008 **
6	0	51.7	34.7	1.4	8.5	1.1	2.6	1.49
	0.02	53.6	35.4	1.4	8.9	0.7	2.2	1.51
	0.2	52.1	35.4	1.3	8.1	0.9	2.3	1.47
	1.0	51.9	35.6	1.3	7.9	1.0	2.6	1.46
	5.0	51.6	36.5	1.4	7.4	1.0	2.1	1.41
	S.E.	0.52	0.27 **	0.05	0.12 **	0.14	0.07	0.01 **
10.5	0	53.6	32.4	1.2	8.1	0.9	3.6	1.65
	0.02	54.1	32.2	1.6	7.8	0.8	3.4	1.68
	0.2	54.3	32.6	1.1	7.8	0.8	3.4	1.67
	1.0	53.8	32.7	1.2	7.8	0.9	3.6	1.65
	5.0	53.0	33.4	1.2	8.2	0.9	3.4	1.59
	S.E.	0.30	0.16 **	0.21	0.08 *	0.09	0.15	0.01 **
21	0	55.0	32.9	1.0	7.9	0.6	2.9	1.67
	0.02	55.3	32.7	1.1	7.6	0.5	2.8	1.69
	0.2	54.8	33.2	1.0	6.9	0.4	2.8	1.65
	1.0	56.0	33.5	0.9	6.7	0.4	2.7	1.67
	5.0	54.9	34.2	1.2	6.3	0	3.4	1.61
	S.E.	0.56	0.45	0.06	0.14 **	0.11	0.14 *	0.03
28.5	0	56.5	32.0	1.0	7.6	0.6	2.6	1.77
	0.02	56.1	32.4	1.0	7.7	0.8	2.6	1.73
	0.2	56.3	33.0	1.1	6.8	0.4	2.7	1.71
	1.0	55.7	33.4	1.0	6.9	0.5	2.7	1.67
	5.0	53.5	31.5	1.1	10.0	0.9	3.2	1.71
	S.E.	0.58 *	0.83	0.05	0.77	0.17	0.13 *	0.05
48	0	58.7	30.8	1.2	5.8	0.9	2.7	1.90
	0.02	55.8	32.0	1.24	7.1	1.0	2.7	1.74
	0.2	55.3	33.1	1.3	6.6	1.0	2.6	1.68
	1.0	56.0	32.9	1.1	6.4	0.9	2.7	1.71
	5.0	54.6	32.7	1.0	8.3	0.8	2.7	1.67
	S.E.	0.70 *	0.54	0.06 *	0.55	0.06	0.13	0.05 *

'*' = significant at 5% level; '**' = significant at 1% level.

Differences determined by analysis of variance.

substantially with an increased incubation period.

The concentrations of the three main VFA, acetic, propionic and butyric acids, increased significantly with a selenate level of 0.02mg/*l* only at 6 hours of incubation, although slight increases were found at most incubation periods. At both three and six hour incubations the concentrations of the main VFA significantly decreased with selenate levels from 0.02 to 5mg/*l*. Although 5mgSe/*l* was the highest selenium level used, results using starch substrate together with extrapolation of the results in table 9, suggest higher selenate additions would further reduce the concentrations of acetic, propionic, iso-butyric and butyric acids, at least at the shorter incubation periods.

The molar proportions calculated from the VFA concentrations are presented in table 10. At incubation periods less than 24h, as the selenate concentration increased above 0.02mg/*l*, % acetic acid decreased slightly, % propionic acid increased significantly and the acetic/propionic acid ratio declined. At 28.5 and 48h periods, % acetic acid decreased significantly as selenate concentrations increased, and the acetic/propionic acid ratio decreased at 48h as selenate levels increased.

Butyric acid molar % slightly declined as selenate levels increased at incubation periods up to 21 h, but the proportions of iso-butyric, iso-valeric and valeric acids showed little or no response to changing selenium concentrations.

CHAPTER 5

DISCUSSION OF RESULTS

5.1 MICROBIAL PROTEIN SYNTHESIS

5.1.1 Differences Between Hay and Starch Substrate Incubations

The lower protein concentration in the rumen liquor used for starch substrate incubations was a result of the removal of much of the protozoa, heavier bacteria and feed protein from the strained rumen liquor (SRL) by centrifugation prior to its inclusion in the incubation mixture.

During 24 hours of incubation, the greater amount of protein synthesised in starch substrate mixtures than in hay substrate mixtures, was a direct result of the starch substrate promoting protein synthesis (Hungate, 1966b) and the absence of microorganisms responsible for protein decomposition which were removed by centrifugation of the SRL (Pearson and Smith, 1943). The presence of these microorganisms in hay substrate mixtures probably contributed to the relatively small amount of protein synthesised. When rumen liquor was not centrifuged, Pearson and Smith (1943) postulated that hydrolysis and synthesis of protein proceeded concurrently, and the hay substrate incubations probably reflect this situation.

5.1.2 The Effect of Selenium on Protein Synthesis

5.1.2.1 Starch Substrate Incubations

The increase in net protein synthesis as the selenate

concentration increased to 0.3mg/*l*, confirms the suggestion by Hidiroglou and Zarkadas (1976) that when selenium was incorporated into bacterial protein it was associated with increased protein synthesis. The increase in synthesis reported here, was also likely to be associated with an increase in bacterial multiplication as Hidiroglou and Lessard (1976) reported low additions of selenium to the rumen increased total bacterial numbers.

However the result is at variance with similar work by Khirwar and Arora (1976) who found no increase in bacterial protein synthesis with added selenium. A ruminant's intake of selenium, prior to the use of its rumen fluid for incubations, was shown by Hidiroglou *et al*. (1968) to diminish protein synthesis when selenium was added to the *in vitro* incubation medium. Khirwar and Arora (1976) recorded the selenium content of forage fed *ad lib*. to the donor animal as 0.08mg/*l*, and as 0.28 and 0.2 mg/*l* in cow and buffalo SRL respectively. These levels in the SRL might have been sufficient to account for the absence of an effect of low amounts of selenium on protein synthesis. The presence of microorganisms responsible for protein degradation in the SRL they used, may have also contributed to selenium having no beneficial effect on protein synthesis.

Khirwar and Arora (1976) reported a drop in protein synthesis above 5mg Se/*l*, and although concentrations declined above 0.3mg Se/*l* in the results reported here, only at selenate levels above 5mg/*l* did protein synthesis drop below that in mixtures containing no selenium. The results suggest

that while selenate levels up to 5 mg/*l* caused an increase in the net amounts of protein synthesised, inhibition of synthesis initially occurs at a much lower selenium concentration (0.3mg/*l*) than previously thought.

5.1.2.2 Hay Substrate Incubations

As the increases in protein synthesis were not significant when selenium was added to the incubation medium, it appears that the hay substrate was responsible for the difference found between hay and starch substrate incubations. The reasons for this are discussed in section 5.5.

The decrease in protein synthesis found when selenate levels exceeded 3mg/*l*, considering the results of Khirwar and Arora (1976), was probably due to selenate inhibiting bacterial multiplication because of indiscriminate substitution of selenium for sulphur and its incorporation into selenoamino acids.

The logarithmic decline found in protein synthesis at high selenate levels may be characteristic of selenium toxicity as Forsberg (1978) found the reduction in fermentation rate was also logarithmically related to high selenium concentrations.

5.2 THE PRODUCTION OF VFA *IN VITRO*

5.2.1 The Differences Between Hay and Starch Substrate Incubations with No Added Selenium

When hay was used as the incubation substrate, the increase in total VFA production as the incubation period increased was fairly linear up to 24 hours, however

the rate of production declined at longer incubation periods probably because of an accumulation of fermentation products in the incubation mixture inhibiting VFA production and/or a decrease in the amount of hay substrate available.

A comparison between the starch substrate VFA concentrations (Table 7) and the hay substrate VFA concentrations at 21 and 28.5 hours (Table 9) when no selenium was added to flasks shows the amounts of VFA produced much higher in flasks containing hay as the substrate. As the donor animal was on a hay diet the microbial population of the rumen fluid used in the *in vitro* incubations would change substantially in experiments where starch was provided as the sole carbohydrate. Because the microbial adaptation to the change in substrate only occurs after a period of time (Warner, 1962), maximum VFA production would not proceed immediately and subsequently VFA concentrations at comparable times would be lower in starch than hay substrate incubation mixtures.

At comparable incubation periods the acetic/propionic acid ratio was lower in the VFA mixtures with starch substrate than in the hay substrate incubation mixtures which is consistent with data from other workers (Hershberger, Bentley, Cline and Tyznik, 1956). Latham, Sharpe and Sutton (1971) showed that readily available carbohydrate increased the proportions of selenomonads, peptostreptococci, lactobacilli and bifidobacteria in rumen fluid. Any increase in numbers of the first two groups, which produce propionic acid from lactic acid, could account for the higher propionic acid molar % and lower acetic/propionic ratio when starch was

used as the substrate.

The differences in VFA production, between hay and starch substrate incubations, indicate the type of substrate affects both numbers and composition of the microbial population.

5.2.2 The Effect of Selenium on VFA Production

5.2.2.1 Starch Substrate Incubations

As the increases in the concentrations of acetic, propionic and butyric acids, with added selenate up to 0.3mg/*l*, paralleled the increase in microbial protein synthesis, and small additions of selenium have been associated with an increase in rumen bacteria numbers (Hidiroglou, Heaney and Jenkins, 1968; Hidiroglou and Lessard, 1976), the elevated production of VFA was probably due to an increase in the numbers of bacteria producing VFA when selenium stimulated bacteria multiplication.

The decrease in iso-butyric acid concentration as selenate levels increased to 0.1mg/*l*, was likely to be a result of an increase in bacterial multiplication, as Allison and Bryant (1963) have shown that iso-butyric acid is converted to valine and assimilated into bacterial protein during protein synthesis.

If an alteration of the VFA metabolic pathways had occurred, a changed pattern of VFA production would have resulted. However the absence of any significant effects on VFA molar % up to a selenate concentration of 0.3mg/*l* tends to support the view that selenium affected only

bacterial numbers, rather than altered the metabolic pathways of carbohydrate fermentation.

Evidence has been presented by Hidiroglou *et al.* (1968) and Hidiroglou and Lessard (1976) that selenium can affect the microbial species composition of rumen fluid. This might explain the changes in VFA molar % when concentrations of selenate exceeded 0.3mg/*l*. As this selenate concentration coincided with a fall in microbial protein synthesis, it suggests that the multiplication of some species of bacteria may have been affected at different selenate concentrations than others. Forsberg (1978) has already found that the concentrations of selenite required to inhibit growth varied widely for the three species of rumen bacteria he studied. Two of the species, *R. albus* and *S. bovis*, are acetic acid producing bacteria, and one, *S. ruminantium*, acetic and propionic acid producing (Hungate, 1966c). Growth inhibition of the propionic acid producing bacteria occurred at a higher selenium concentration than that needed to inhibit the other two species.

It is postulated that in the experiments reported here, the reduction in % acetic acid, from 0.3 to 2mg Se/*l*, was a result of selenate inhibiting acetic acid producing bacteria but continuing to promote the growth of propionic acid producing organisms. When selenate concentrations exceeded 2mg/*l*, the decrease in propionic acid production (and the associated increase in acetic/propionic ratio), may have been a result of a decrease in the multiplication of propionic producing bacteria by selenate. The inhibition of progressively more species of bacteria as selenate levels exceeded

0.3mg/*l*, would result in the observed decline in total VFA concentration

5.2.2.2 Hay Substrate Incubations

Although significant increases in VFA concentrations were only found at 6 h incubation when the selenate level was 0.02mg/*l*, slight increases in VFA at this selenate concentration were found at all incubation periods. This may indicate that low amounts of selenium can increase VFA production when hay is used as the substrate, but the results lead one to accept this conclusion with reservations.

The decrease in total VFA concentrations above selenate concentrations of 0.2mg/*l*, is similar to that found in the starch substrate incubations with selenate levels above 0.3mg/*l*, and probably due to selenium inhibiting the activity or multiplication of the bacteria. The acetic/propionic acid ratio did not increase from 1 to 5mg Se/*l* however, as it did in the starch substrate incubations. This was probably a result of the different microbial populations sustained by the two substrates and, as discussed in section 5.5, effectively less selenium may have been available to be utilised by the bacteria in the hay substrate incubations. If this were the case one would expect selenate concentrations above 5mg/*l* to increase the ratio.

5.3 THE CHANGE IN pH OF THE INCUBATION MIXTURE WITH STARCH SUBSTRATE

The significant drop in pH when selenate concentrations increased to 0.1mg/*l* was almost certainly due to an increase

in the production of all fermentation acids up to this selenate level and confirms the *in vivo* result of Farzaliev (1981). To some extent, the increase in VFA produced up to 0.1mg Se/*l* contributed towards the decline in pH.

The large increase in pH from a selenate concentration of 1 to 10mg/*l* cannot be due solely to the decline in fermentation acid production because total VFA produced at 10mg Se/*l* is about the same as that with no selenium added, yet the pH at 10mg Se/*l* (7.2) is substantially higher than that (pH 4.8) with no additional selenium.

Production of ammonia from urea does not depend on active or growing bacteria (Howard, 1984), and the visual observation that flasks containing high levels of selenium contained a rapidly sedimenting suspension may be indicative of large numbers of dead bacteria. With ammonia production continuing at high selenium concentrations, but less ammonia being utilised, as protein synthesis declined at the high selenium levels, the increase in pH was likely to be due to an accumulation of ammonia.

5.4 THE EFFECT OF SELENIUM ON MICROBIAL DIGESTION

An increase in the fermentation of starch was shown by Hunt *et al.* (1954) to increase both total VFA production and protein synthesis and decrease the pH of the incubation mixture. From the results of starch substrate incubations reported here, that showed selenate concentrations up to 0.3mg/*l* simultaneously increased VFA production and protein synthesis and caused a drop in pH, it is most likely that

selenium would also stimulate the fermentation of starch up to 0.3mg/*l*.

When hay was used as the carbohydrate substrate a slight drop in % digested occurred from 0 to 0.2mg Se/*l*. Although the slight increase in protein concentration in hay substrate mixtures containing up to 3mg Se/*l* was not statistically significant, Hidiroglou and Lessard (1976) have demonstrated that small doses of selenium can increase microbial numbers. If in fact an increase in microbial numbers had occurred up to a selenium concentration of 0.2mg Se/*l*, the extra bacterial weight associated with the hay particles (Warner, 1962) would be weighed as part of the residue and interpreted as a drop in % hay digested. Although there is no direct evidence for this explanation, it is unlikely that the drop in % hay digested was caused solely by a decrease in bacterial activity as both Little *et al*. (1958) and Martinez and Church (1970) reported no change in % cellulose digested up to a selenium concentration of 0.3mg Se/*l*.

As found by the last two authors and Billon *et al*. (1975), selenium concentrations above 0.4mg/*l* depressed microbial digestion. This was probably due to a decreased ability of bacteria to digest the hay substrate and/or a reduction in the numbers of bacteria in the incubation mixture. As protein synthesis declined only above 4mg Se/*l*, and presumably bacterial multiplication also, probably a reduction in the ability of microorganisms to digest organic matter resulted before multiplication was affected.

5.5 THE DIFFERENCES IN THE EFFECTS OF SELENATE BETWEEN HAY AND STARCH SUBSTRATE INCUBATIONS

When hay was used as the incubation substrate, few significant differences were found in protein synthesis, VFA production and dry matter digestion at the low selenium concentrations where marked changed occurred in the starch substrate mixtures. Ellis, Pfander, Muhrer and Pickett (1958) reported stimulation by molybdenum of bacterial digestion was dependant on the substrate used, and the effects of other trace elements on VFA production by rumen bacteria were found by Uesaka, Kawashima and Zembayashi (1966) to differ with the type of carbohydrate in the *in vitro* medium because of the altered microbial populations. The carbohydrate levels of the diet have been shown by Whanger *et al.* (1968) to affect the amount of inorganic selenium to be converted to unavailable forms, presumably also because of the change in microbial population.

In the experiments reported here the possibility exists that the type of microbial population sustained by the hay substrate caused more of the added selenate to be reduced to unavailable forms and less available for bacterial metabolism. The end result would be less selenium incorporated into bacterial protein, and as the effects of selenium on bacterial metabolism are associated with this incorporation (Hidiroglou and Zarkadas, 1976), low concentrations of selenium would have less influence on bacterial metabolism in the hay substrate incubations than in the starch substrate incubations.

The presence of selenium (as selenoamino acids) in the hay substrate (Peterson and Butler, 1962) and in the non microbial material and protozoa of the SRL, (Whanger *et al.*, 1978) added to the incubation mixture with hay substrate, would contribute little to the total selenium concentration of the incubation mixture. Protozoa and non-microbial material have a relatively low selenium content compared to bacteria (Whanger *et al.*, 1978) and the hay substrate (0.06mg Se/kg dry matter) would contribute only 0.03μg Se to each flask. However Hidiroglou *et al.* (1968) and Paulson *et al.* (1968) found the organic form of selenium could be metabolised differently than inorganic selenium. Therefore the organic form of selenium, present in these materials, may have satisfied the selenium requirement of the microorganisms in the hay substrate incubation mixtures, the added selenate having little effect at low concentrations.

5.6 SUMMARY

In the experiments reported here, inorganic selenium (as sodium selenate) up to a concentration of 0.3 mg Se/*l* was shown to increase bacterial VFA production and protein synthesis and lower the pH when starch was used as the carbohydrate substrate for *in vitro* incubations. This was thought to be a result of an increase in bacterial multiplication concomitant with the incorporation of selenium into bacterial proteins.

When hay was used as the substrate, slight increases in VFA production and protein synthesis were noted with low selenate concentrations, however the increases in VFA

production were not significant at all but one incubation period and the increase in protein synthesis was not significant. It was postulated that the differences found between hay and starch substrate incubations, at the low selenium concentrations, were a result of the hay substrate causing the bacteria to reduce more of the selenium to unavailable forms, which were unable to be reutilised by the bacteria.

At selenate concentrations above 0.3mg/*l*, VFA production and protein synthesis declined but the proportions of VFA produced altered which suggested that some species of bacteria were inhibited at lower selenium concentrations than others. Declines were also found in VFA production, protein synthesis and also dry matter digestion using hay as the carbohydrate at higher selenate concentrations. The inhibition of bacterial metabolism might have been due to indiscriminate substitution of selenium for sulphur in bacterial proteins, but further research is needed to determine this, perhaps using pure strains of bacteria.

The results, using starch as the incubation substrate, demonstrated that while selenate concentrations could range from 0 to 10mg/*l* without causing VFA production or protein synthesis to decline below the levels with no selenium addition, a narrow boundary separated a stimulatory from an inhibitory selenium concentration. While *in vitro* incubations can only give an indication of the *in vivo* situation, it appears a minimum bacterial requirement of around 0.3mg/*l* is necessary for optimum bacterial metabolism. While a minimum dietary selenium concentration has been documented (0.03mg/kgDM) for the animal, the resulting rumen selenium concentration

is unknown and further work is needed to correlate the selenium intake with the actual rumen concentration. The results here suggest the rumen selenium concentration may be critical for optimum bacterial activity.

APPENDICES

Appendix 1 : A comparison between two centrifugation speeds to precipitate bacterial protein.

The tungstic acid method of precipitating bacterial protein used in section 3.2.1 involved long periods of standing the mixture prior to centrifugation at 2000 x g for 20 minutes. Hidiroglou and Zarkadas (1976) precipitated the bacterial fraction prior to precipitating the bacterial protein by centrifuging at 20 000 x g for 30 minutes. To shorten the standing time in the tungstic acid method, the mixture was centrifuged at 20 000 x g for ten minutes after a 10 minute standing period, and washed in the usual manner between centrifugations.

The protein content was determined as in section 3.2.2 for protein precipitated by both centrifugation methods. The results in table 11 show no significant differences in the protein concentration of SRL precipitated by the two methods.

The method involving centrifugation at 2000 x g was used for all samples in these experiments.

Table 11: A comparison between two methods of bacterial protein sample preparation

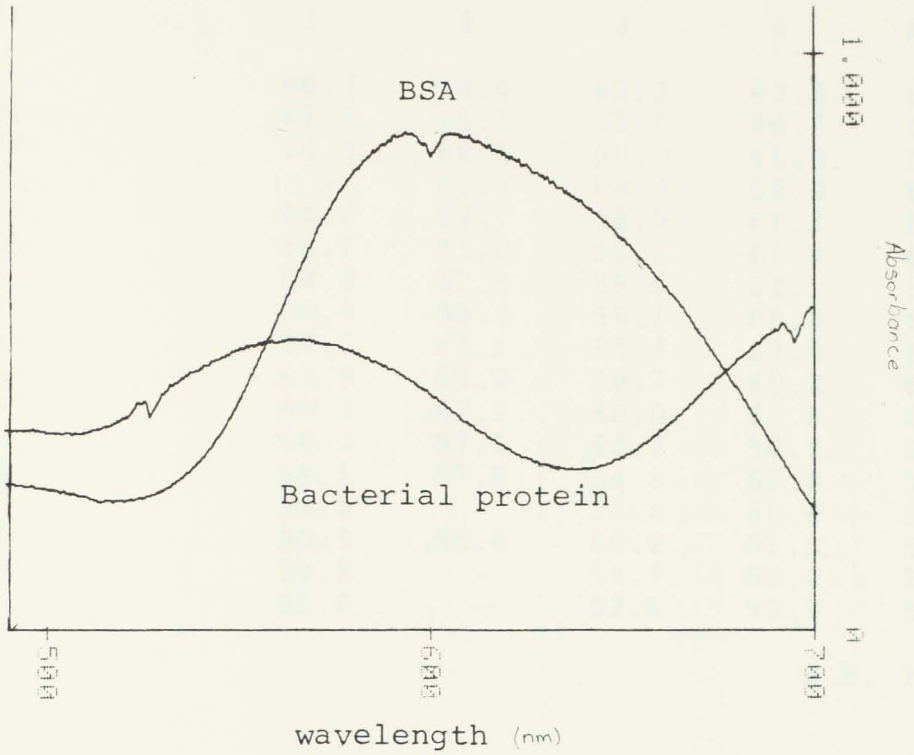
Centrifugation force (xg)	Protein concentration of SRL (mg/l) 1	2	3	MEAN	S.E.
2000	564.4	590.2	561.6	572.1	7.9
20000	565.9	589.7	596.9	584.2	8.1

Appendix 2 : An evaluation of two methods to measure protein concentrations.

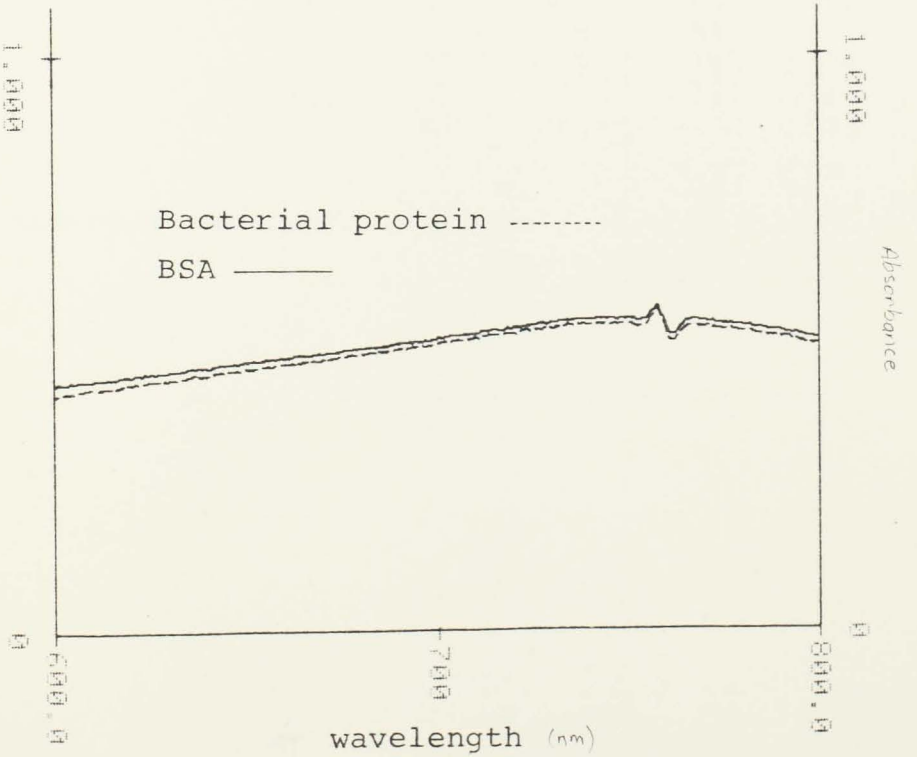
The Coomassie Brilliant Blue G assay for proteins described by Bradford (1976) was evaluated for the estimation of microbial protein as the method has several advantages over the standard Lowry procedure (Spector, 1978). A wavelength scan of solution containing bovine serum albumin confirmed a peak at 595nm, but a scan of solution containing precipitated bacterial protein had a peak at 565nm (shown in figure 10a). Measuring samples at 595nm produced large variations in protein concentration, perhaps because of interfering substances present in the solution. Further purification of bacterial protein may overcome this problem. Using the Lowry method the wavelength scan of solutions of BSA and bacterial protein are reproduced in figure 10b.

Figure 10 : Wavelength scans of standard BSA and bacterial protein, using the Coomassie Brilliant Blue G assay and Lowry assay.

a) Coomassie Brilliant Blue G assay



b) Lowry assay



Appendix 3 : The effect of selenate on the digestion of a standard hay substrate.

Selenate Concentration (mg/l)	% Dry Matter digested				
	1	2	3	4	MEAN
0	66.7	64.4	63.3	63.3	64.4
0.01	61.8	66.7	61.1	66.7	64.1
0.02	64.6	60.2	60.0	60.0	61.2
0.03	-	61.3	62.0	62.0	61.8
0.04	64.4	64.7	58.9	60.2	62.1
0.15	58.7	61.0	60.6	60.2	60.1
0.2	58.2	57.3	59.6	63.1	59.6
0.3	60.4	58.4	59.1	60.9	59.7
0.4	59.7	61.1	64.4	62.0	61.8
0.5	62.9	60.2	60.7	60.2	61.0
0.6	60.3	62.2	60.0	61.8	61.1
1.0	60.2	57.3	60.4	58.2	59.1
1.5	59.1	57.8	54.9	60.0	58.0
2.0	54.6	-	57.6	60.0	57.4
3.0	50.9	55.6	60.2	51.8	54.6
4.0	53.3	-	54.7	52.0	53.3
5.0	51.6	-	52.6	53.3	52.5

S.E. 1.08

Appendix 4 : The effect of selenium on protein concentrations after 24 hours of incubation with a hay substrate

Selenate concentration (mg/l)	Protein concentration (mg/l) 1	2	3	4	MEAN
Pre Incubation concentration	105	126	118	108	114
0	142	121	114	108	121
0.005	133	178	97	98	126
0.01	158	126	110	161	138
0.02	162	111	176	-	150
0.06	168	161	129	124	145
0.08	174	161	126	154	154
0.12	177	114	114	146	138
0.15	156	140	159	120	144
0.20	95	-	166	147	136
0.25	164	110	172	132	145
0.3	164	166	158	112	150
0.4	151	146	139	158	148
0.6	117	182	114	144	139
0.8	189	153	106	163	153
1.0	106	145	-	161	137
1.2	133	148	-	150	144
1.5	119	164	107	165	138
2.0	153	113	117	168	138
2.5	167	177	115	149	152
3	163	166	156	177	165
4	192	152	120	114	145
6	147	166	-	120	144
8	139	137	134	102	128
10	110	151	129	113	126
12	152	125	104	122	125
15	86	115	148	100	112
20	109	-	99	111	106
25	110	109	83	107	102
30	83	88	96	96	90
40	67	84	79	76	76
60	87	85	72	77	80
80	70	67	81	-	73

Standard error (S.E.) = 11.9

Appendix 5 : The effect of selenium on the net amount of protein synthesised during a 24h incubation with a starch substrate.

Selenate concentration (mg/l)	Protein concentration (mg/l)					
	1	2	3	4	5	MEAN
0	125	149	95	185	233	157.9
0.01	199	234	188	228	190	208.1
0.05	269	155	251	267	253	239.9
0.3	258	284	218	265	251	255.1
0.5	266	293	221	199	276	251.1
1.0	244	240	277	207	247	242.9
2.0	216	193	219	234	217	220.9
3.0	220	200	205	224	198	209.4
5.0	169	153	202	127	188	167.9
7.0	152	126	130	125	110	128.6
10.0	42	85	72	75	89	
	86	66	79	71	65	72.3

S.E. = 14.1 using analysis of variance

Pre-Incubation Level						
	147	143	148	157	150	149.2 ± 5.2

Appendix 6 : The effect of selenium on the acidity of the incubation mixture with a starch substrate.

Selenate concentration (mg/l)	pH of incubation mixture					
	1	2	3	4	5	MEAN
0	4.7	4.8	4.8	4.8	4.8	4.78
0.01	4.85	4.8	4.8	4.75	4.75	4.79
0.05	4.8	4.8	4.7	4.65	4.65	4.72
0.1	4.6	4.6	4.6	4.65	4.6	4.61
0.3	4.6	4.7	4.7	4.6	4.6	4.64
0.5	4.6	4.65	4.65	4.7	4.7	4.66
1.0	4.7	4.6	4.6	4.7	4.7	4.66
2.0	4.95	4.8	4.9	4.9	4.9	4.89
3.0	5.2	5.4	5.3	5.25	5.25	5.28
5.0	6.8	6.5	6.85	7.1	6.8	6.81
7.0	6.8	6.9	7.15	6.85	7.05	6.95
10.0	7.35	7.3	7.3	7.4	7.0	7.27

Standard Error = 0.05 determined by analysis of variance.

Appendix 7 : The effect of selenium on the production of VFA during a 24h incubation with starch substrate

Selenate Concentration (mg/l)	VFA concentrations (mg/l)						
	ACET.	PROP.	I-BUT.	BUT.	I-VAL.	VAL.	TVFA
	26.3	13.5	2.6	0.3	1.7	0.0	44.4
	36.3	22.3	6.0	1.4	0.0	-1.3	64.7
	45.2	27.3	7.3	3.4	1.0	-0.8	83.5
0	43.3	27.3	7.0	2.9	1.5	5.6	87.6
	37.3	24.3	6.0	1.3	-	-	68.9
	39.3	19.3	4.0	0.0	-	-	62.6
	46.3	21.3	5.4	1.7	-	-	74.7
0.01	45.3	25.3	5.1	2.3	-	-	78.0
	71.3	40.3	4.1	4.6	-0.1	1.3	121.5
	50.3	26.3	4.5	1.6	-0.5	0.4	82.6
0.05	57.3	34.3	4.1	2.5	1.2	0.7	100.1
	65.3	37.3	6.3	4.3	-0.3	0.2	113.1
	69.3	42.3	4.9	4.2	-0.5	-0.3	119.9
	63.3	41.3	4.7	6.8	0.4	-0.4	116.1
0.1	61.3	35.3	1.9	5.6	0.4	1.4	105.9
	75.3	44.3	6.7	6.2	2.6	0.9	136.0
	71.3	57.3	7.8	10.0	0.7	0.9	148.0
	98.3	59.3	7.4	10.0	2.4	1.1	178.5
0.3	86.3	42.3	7.2	6.8	0.1	1.0	143.7
	68.3	35.3	6.0	13.3	0.0	-0.3	122.6
	76.3	43.3	11.8	15.7	0.0	0.7	147.8
	61.3	53.3	8.5	6.4	3.5	1.6	134.6
0.5	89.3	64.3	10.5	11.4	2.0	1.3	178.8
	82.2	74.1	6.8	10.4	1.6	1.7	176.8
	63.3	43.3	5.8	8.9	7.8	4.5	133.6
1.0	66.8	41.1	4.0	8.3	0.0	1.1	121.6
	66.3	57.3	2.7	9.2	-	-	135.5
	77.3	64.3	2.7	8.7	-	-	153.0
2.0	61.3	53.3	1.6	10.7	-	-	126.9
	61.3	43.3	2.1	6.7	-0.8	-0.3	112.3
	79.3	59.3	2.9	11.1	0.4	0.9	153.9
	80.3	45.3	0.5	13.9	-0.2	-0.3	139.5
3.0	73.3	70.3	2.3	9.6	1.5	-0.1	156.9
	69.3	38.3	1.3	7.1	-0.3	1.7	117.4
	52.3	30.3	0.8	4.7	2.1	1.7	91.9
	80.3	46.3	0.9	7.9	0.0	0.6	136.0
5.0	56.3	29.3	1.0	4.3	0.0	0.0	90.9
	51.3	28.3	-0.3	3.2	0.5	0.5	83.5
	66.3	40.3	1.5	7.9	1.2	2.0	119.2
	55.5	29.3	1.4	5.6	1.7	1.9	95.2
7.0	49.3	25.3	1.4	5.6	1.7	1.8	85.1
	38.3	18.3	0.5	0.4	1.0	1.7	60.2
	41.3	21.3	2.6	1.9	1.5	1.7	70.3
	42.3	22.3	0.8	1.7	0.8	0.5	68.4
10.0	40.3	16.3	1.0	0.6	1.8	0.7	60.7

Appendix 8 : The effect of selenium on the proportions of VFA produced during a 24h incubation with a starch substrate

Selenate Concentration (mg/l)	VFA molar %						
	ACET.	PROP.	I-BUT.	BUT.	I-VAL.	VAL.	AC/PR
0	59.23	30.40	5.85	0.67	3.82	0.00	1.94
	56.10	34.46	9.27	2.16	0.00	-2.00	1.62
	54.25	32.69	8.74	4.07	1.19	-0.95	1.65
	49.42	31.16	7.99	3.31	1.71	6.39	1.58
0.01	54.13	35.26	8.70	1.88	-	-	1.53
	62.77	30.83	6.38	0.00	-	-	2.03
	61.98	28.51	7.22	2.27	-	-	2.17
	58.07	32.43	6.53	2.94	-	-	1.79
0.05	58.68	33.16	3.37	3.78	-0.08	1.06	1.76
	60.89	31.84	5.44	1.93	-0.60	0.48	1.91
	57.24	34.26	4.09	2.49	1.19	0.69	1.67
0.1	57.73	32.97	5.57	3.80	-0.26	0.17	1.75
	57.79	35.27	4.08	3.50	-0.41	-0.25	1.63
	54.52	35.57	4.04	5.85	0.34	-0.34	1.53
	57.88	33.33	1.79	5.28	0.37	1.32	1.73
0.3	55.36	32.57	4.92	4.55	1.91	0.66	1.69
	48.17	38.71	5.27	6.75	0.47	0.60	1.24
	55.07	33.22	4.14	5.60	1.34	0.61	1.65
	60.05	29.43	5.01	4.73	0.06	0.69	2.04
0.5	55.70	28.79	4.89	10.84	0.00	-0.24	1.93
	51.62	29.29	7.98	10.62	0.00	0.47	1.76
	45.54	39.59	6.31	4.75	2.60	1.18	1.15
	49.94	35.96	5.87	6.37	1.11	0.72	1.38
1.0	46.49	41.91	3.84	5.88	0.90	0.96	1.10
	47.38	32.41	4.34	6.66	5.83	3.36	1.46
	54.93	34.04	3.28	6.82	0.00	0.90	1.61
2.0	48.92	42.28	1.99	6.78	-	-	1.15
	50.52	42.02	1.76	5.68	-	-	1.20
	48.30	42.00	1.26	8.43	-	-	1.15
3.0	54.58	38.55	1.86	5.96	-0.71	-0.26	1.41
	51.52	38.53	1.88	7.21	0.25	0.58	1.33
	57.56	32.47	0.35	9.96	-0.14	-0.21	1.77
	46.71	44.80	1.46	6.11	0.95	-0.06	1.04
5.0	59.02	32.62	1.10	6.04	-0.25	1.44	1.80
	56.90	32.97	0.87	5.11	2.28	1.84	1.72
	59.04	34.04	0.66	5.80	-0.00	0.44	1.73
	61.93	32.23	1.10	4.73	0.00	0.00	1.92
7.0	61.43	33.89	-0.35	3.83	0.59	0.59	1.81
	55.62	33.80	1.25	6.62	1.67	1.67	1.64
	58.08	30.77	1.47	5.88	1.99	1.99	1.88
	57.93	29.72	1.64	6.58	2.11	2.11	1.94
10.0	63.62	30.39	0.83	0.66	1.66	2.82	2.09
	58.74	30.29	3.69	2.70	2.13	2.41	1.93
	61.84	32.60	1.16	2.48	1.16	0.73	1.89
	66.39	26.85	1.64	0.98	2.96	1.15	2.47

Appendix 9 : The effect of selenium on the concentrations of VFA following incubation with hay substrate

Incubation Period (h)	Selenate Concentration (mg/l)	VFA concentrations (mg/l) Acet.	Prop.	I-But.	But.	I-Val.	Val.	TVFA
	0	72.4	47.5	2.2	11.7	1.1	1.7	137
		70.5	44.8	2.2	11.0	0.9	1.7	131
		64.0	41.6	2.1	10.1	1.6	2.4	122
		67.4	42.8	2.3	10.6	1.5	2.6	127
	0.02	75.4	48.0	2.4	11.8	0.9	2.1	140
		71.0	45.0	2.2	11.0	1.7	2.7	134
		70.0	44.9	2.1	11.1	1.7	3.3	133
		66.4	42.4	2.2	10.3	1.8	2.6	127
3	0.2	70.6	44.7	2.2	11.3	1.6	2.3	133
		68.0	42.8	2.1	10.7	1.7	2.2	127
		69.8	43.8	2.1	11.0	1.7	2.2	131
		70.8	45.1	2.2	11.1	1.7	2.3	133
	1.0	70.4	46.1	2.2	11.1	0.8	1.8	133
		68.2	43.6	2.1	10.8	1.7	2.6	129
		67.2	43.3	2.2	10.5	1.7	2.4	127
	5.0	65.1	43.6	2.1	3.0	0.8	1.4	123
		64.9	44.0	2.1	10.4	1.5	1.5	124
		62.1	41.8	2.0	10.3	1.8	2.2	120
		61.4	41.0	1.8	10.0	0.8	1.2	116
	0	80.5	53.6	1.9	13.7	1.7	4.4	156
		84.9	56.7	2.3	13.9	1.8	4.2	164
		85.6	58.3	2.5	13.8	1.8	4.4	166
		84.7	57.1	2.4	13.8	1.8	4.2	164
	0.02	90.1	60.4	2.4	14.6	1.4	4.2	173
		104.4	68.4	2.6	17.7	-	4.3	197
		95.3	61.8	2.6	16.0	1.1	3.7	168
		92.6	62.1	2.3	15.4	-	3.8	176
6	0.2	86.1	57.6	2.2	13.4	1.7	3.8	165
		90.6	62.0	2.2	14.2	1.7	4.1	175
		86.3	58.4	2.3	13.3	1.7	4.1	166
		91.5	63.2	2.3	14.1	0.8	3.3	175
	1.0	82.7	56.6	2.3	12.8	2.0	4.2	160
		83.0	57.9	2.2	12.8	2.1	4.1	162
		88.0	59.6	2.0	13.1	0.5	4.2	167
	5.0	89.1	61.8	2.5	12.7	1.1	3.8	171
		88.8	62.7	2.2	12.9	1.7	3.5	172
		85.9	61.3	2.2	12.5	1.8	3.6	167
		80.5	57.5	2.3	11.4	1.8	3.1	157

Appendix 9 (cont.)

	0	108.4	65.6	2.4	16.7	1.8	6.9	202
		111.8	67.5	2.5	16.5	1.9	7.1	208
		109.8	66.7	2.6	16.6	1.9	8.0	206
	0.02	110.8	66.1	6.1	16.0	2.1	7.3	208
		110.3	66.2	2.3	16.0	1.7	7.5	204
		129.2	76.5	2.6	18.1	1.2	7.1	235
		113.8	67.4	2.5	16.6	2.0	7.1	210
10.5	0.2	114.4	69.3	2.6	16.3	2.0	7.5	212
		117.7	70.5	2.4	16.9	0.9	6.5	215
		107.7	64.8	2.4	15.7	1.8	6.9	199
		115.7	68.9	2.2	16.9	1.6	7.7	213
	1.0	109.0	67.0	2.6	15.8	2.0	7.8	204
		109.6	67.4	2.5	16.3	1.9	7.9	206
		116.3	69.1	2.4	16.6	0.9	6.5	212
		112.7	68.3	2.3	16.4	1.8	8.0	209
	5.0	108.5	65.6	2.4	15.8	1.5	7.5	201
		108.6	69.8	2.5	16.6	2.1	6.7	206
		107.5	67.8	2.4	16.9	2.0	6.6	203
		106.9	68.3	2.4	17.3	2.0	6.9	204
21	0	174	105	2.4	25.9	1.0	7.9	318
		151	90	2.7	21.4	-	7.3	272
		146	87	2.8	20.3	2.4	9.1	269
		152	92	3.0	21.9	1.3	8.5	279
	0.02	149	89	3.1	20.8	1.40	8.0	273
		178	106	2.8	25.6	-	7.9	321
		145	86	3.1	19.8	1.5	8.1	263
		173	101	3.1	22.9	1.0	8.9	311
	0.2	189	117	3.1	26.3	1.1	8.8	364
		174	105	3.0	21.7	-	8.6	312
		160	98	2.7	20.4	1.3	8.9	292
		153	91	3.0	17.9	-	8.0	273
	1.0	170	103	2.8	21.4	1.2	8.7	308
		160	95	2.4	18.3	-	7.8	283
		165	101	3.0	20.2	-	8.1	298
		198	115	3.1	22.5	-	8.1	347
	5.0	179	99	4.5	20.8		11.7	315
		144	93	2.8	18.0	0	8.7	266
		128	83	2.5	14.3	0	7.6	236
		128	83	2.8	14.6	0	7.8	236

Appendix 9 (cont.)

	0	211	107	3.2	24.4	1.7	8.3	356
		193	116	3.3	28.3	-	8.9	350
		189	112	3.4	28.9	2.2	8.7	345
		172	98	3.3	22.0	2.0	8.8	305
28.5	0.02	213	126	3.3	30.9	-	9.1	384
		172	98	3.4	21.7	2.5	9.4	308
		185	106	3.7	27.1	-	8.5	330
		196	112	3.3	25.4	-	8.0	345
	0.2	179	106	3.3	33.2		8.4	321
		182	106	3.5	22.3	1.3	8.5	324
		175	103	3.3	20.4		8.5	311
		166	95	3.3	18.4		8.2	291
	1.0	184	108	3.4	21.2	1.3	8.8	327
		203	126	3.4	27.5	2.4	9.3	372
		165	98	3.3	18.5	1.2	8.7	296
		190	114	3.4	24.8	-	8.8	340
	5.0	166	107	2.8	21.5	-	10.1	308
		220	116	5.3	49.0	2.8	12.0	407
		184	102	4.7	47.7	5.7	13.0	357
		159	100	3.0	22.0	1.6	8.6	296
	0	223	117	4.6	22.3	3.6	9.7	380
		206	109	4.2	21.6	3.2	9.4	353
		207	108	4.3	19.0	3.1	10.4	353
48	0.02	231	130	4.9	30.8	3.7	11.0	411
		174	106	4.3	19.8	3.5	9.5	317
		243	145	5.4	39.0	4.6	11.0	449
		209	111	4.4	22.3	3.5	10.4	361
	0.2	210	114	4.3	21.1	4.6	10.1	363
		148	96	4.3	13.2	2.9	7.7	273
		262	158	5.5	38.1	4.2	11.5	480
		246	148	5.2	35.9	3.7	10.6	449
	1.0	188	104	3.7	17.4	2.8	9.1	325
		232	135	4.4	27.7	3.6	10.3	412
		181	114	3.7	22.7	2.9	10.9	336
		234	137	4.4	28.5	3.5	10.5	418
	5.0	235	142	3.9	31.3	3.2	10.9	427
		253	145	4.8	43.8	4.0	13.1	464
		209	128	3.7	30.9	3.0	10.4	385

Acet. = acetic acid, Prop. = propionic acid, I-But. = iso-butyric acid, But. = butyric acid, I-Val. = iso-valeric acid, Val. = valeric acid, TVFA = total VFA.

Appendix 10 : The effect of selenium on the proportions of VFA after incubation with a hay substrate

Incubation Time (h)	Selenate Concentration (mg/l)	VFA molar %						
		Acet.	Prop.	I-But.	But.	I-Val.	Val.	Ac/Pr
3	0	52.84	34.67	1.60	8.54	0.80	1.24	1.52
		53.81	34.19	1.67	8.39	0.68	1.29	1.57
		52.45	34.09	1.72	8.27	1.31	1.96	1.53
		53.02	33.67	1.80	8.33	1.18	2.04	1.57
		53.47	34.60	1.70	8.36	0.68	1.48	1.54
	0.02	52.59	33.92	1.62	8.14	1.28	2.00	1.55
		52.23	34.10	1.56	8.28	1.26	2.46	1.53
		51.84	34.32	1.76	8.24	1.44	2.08	1.51
		53.03	33.60	1.72	8.52	1.22	1.72	1.57
		53.24	33.58	1.70	8.46	1.36	1.79	1.58
	0.2	53.33	33.50	1.66	8.50	1.33	1.75	1.59
		53.19	33.93	1.72	8.36	1.31	1.80	1.56
		52.93	34.66	1.65	8.34	0.66	1.35	1.52
	1.0	52.86	33.79	1.62	8.37	1.32	2.01	1.56
		52.91	34.09	1.73	8.26	1.33	1.88	1.55
		52.92	35.44	1.70	2.43	0.69	1.13	1.49
		52.33	35.48	1.69	8.38	1.20	1.20	1.47
	5.0	51.75	34.83	1.66	8.58	1.50	1.83	1.48
		52.81	35.26	1.55	8.60	0.69	1.08	1.49
6		51.60	34.35	1.21	8.78	1.08	2.69	1.50
	0	51.76	34.57	1.40	8.47	1.09	2.56	1.49
		51.56	35.12	1.50	8.31	1.08	2.65	1.46
		51.64	34.81	1.46	8.41	1.09	2.56	1.48
		52.08	34.91	1.38	8.43	0.80	2.42	1.49
		52.99	34.72	1.31	8.98	-	2.18	1.52
	0.02	56.72	36.78	1.54	9.52	0.65	2.20	1.54
		52.61	35.28	1.30	8.75	-	2.15	1.49
		52.18	34.90	1.33	8.12	1.03	2.30	1.49
		51.77	35.42	1.25	8.11	0.97	2.34	1.46
	0.2	51.98	35.18	1.38	8.01	1.02	2.46	1.47
		52.28	36.11	1.31	8.05	0.48	1.90	1.44
		51.68	35.37	1.43	8.00	1.25	2.62	1.46
	1.0	51.23	35.74	1.35	7.90	1.29	2.53	1.43
		52.69	35.68	1.19	7.84	0.32	2.51	1.47
		52.10	36.14	1.46	7.42	0.64	2.22	1.44
		51.62	36.45	1.27	7.50	0.98	2.03	1.41
	5.0	51.43	36.70	1.31	7.48	1.07	2.15	1.40
		51.27	36.62	1.46	7.26	1.14	1.97	1.40

10.5		53.66	32.47	1.18	8.26	0.89	3.41	1.65
	0	53.75	32.45	1.20	7.93	0.91	3.41	1.65
		53.30	32.37	1.26	8.05	0.92	3.88	1.64
		53.26	31.77	2.93	7.69	1.00	3.50	1.67
	0.02	54.06	32.45	1.12	7.84	0.83	3.67	1.66
		54.97	32.55	1.10	7.70	0.51	3.02	1.68
		54.19	32.09	1.19	7.90	0.95	3.38	1.68
		53.96	32.68	1.22	7.68	0.94	3.53	1.65
		54.74	32.79	1.11	7.86	0.41	3.02	1.66
	0.2	54.12	32.56	1.20	7.88	0.90	3.46	1.66
		54.31	32.34	1.03	7.93	0.75	3.61	1.67
		53.43	32.84	1.27	7.74	0.98	3.82	1.62
		53.20	32.72	1.21	7.91	0.92	3.83	1.62
	1.0	54.85	32.59	1.15	7.83	0.46	3.06	1.68
		53.79	32.60	1.09	7.82	0.85	3.81	1.65
		53.98	32.63	1.19	7.86	0.74	3.73	1.65
	5.0	52.71	33.88	1.21	8.05	1.01	3.25	1.55
		52.95	33.39	1.18	8.32	0.98	3.25	1.58
		52.40	33.48	1.17	8.48	0.98	3.38	1.56
21	0	55.00	33.08	0.77	8.14	0.31	2.48	1.66
		55.51	33.08	0.99	7.86	-	2.68	1.67
		54.60	32.34	1.04	7.54	0.89	3.38	1.68
		54.69	32.97	1.07	7.84	0.46	3.04	1.65
		54.90	32.60	1.13	7.61	0.51	2.93	1.68
	0.02	55.45	33.02	0.87	7.97	-	2.48	1.67
		55.13	32.69	1.17	7.52	0.57	3.07	1.68
		55.85	32.47	0.99	7.36	0.32	2.86	1.71
		52.14	32.14	0.85	7.22	0.30	2.41	1.61
	0.2	55.76	33.65	0.96	6.95	-	2.75	1.65
		55.10	33.56	0.92	6.98	0.44	3.04	1.64
		52.26	33.33	1.09	6.55	-	2.93	1.68
		55.19	33.44	0.90	6.94	0.38	2.82	1.65
	1.0	56.53	33.56	0.84	6.46	-	2.75	1.68
		55.36	33.89	1.00	6.77	-	2.71	1.63
		57.06	33.14	0.89	6.48	-	2.33	1.72
		56.82	31.42	1.42	6.60	0	3.71	1.80
	5.0	54.13	34.96	1.05	6.76	0	3.27	1.54
		54.23	35.16	1.05	6.05	0	3.22	1.54
		54.23	35.16	1.18	6.18	0	3.30	1.54

28.5	0	59.52	30.00	0.89	6.85	0.47	2.33	1.98
		55.28	33.14	0.94	8.08	-	2.54	1.66
		54.89	32.66	0.98	8.37	0.63	2.52	1.68
		56.32	32.16	1.08	7.21	0.65	2.88	1.75
	0.02	55.65	32.94	0.88	8.04	-	2.36	1.68
		55.94	31.94	1.10	7.04	0.81	3.05	1.75
		56.06	32.12	1.12	8.21	-	2.57	1.74
		56.84	32.46	0.96	7.36	-	2.31	1.75
	0.2	55.95	33.08	1.02	7.22	-	2.61	1.69
		56.17	32.71	1.08	6.88	0.40	2.62	1.71
		56.27	33.31	1.06	6.55	-	2.73	1.68
		56.94	32.85	1.13	6.32	-	2.81	1.73
	1.0	56.26	33.02	1.03	6.48	0.39	2.69	1.70
		54.56	33.87	0.91	7.39	0.64	2.50	1.61
		55.94	33.20	1.11	6.25	0.40	2.93	1.68
		55.88	33.52	1.00	7.29	-	2.58	1.66
	5.0	54.09	34.80	0.90	6.98	-	3.27	1.55
		54.05	28.59	1.30	12.03	0.68	2.94	1.89
		51.70	28.57	1.31	13.36	1.59	3.64	1.80
		53.98	34.02	1.01	7.43	0.54	2.90	1.58
48	0	58.84	30.78	1.21	5.86	0.94	2.55	1.911
		58.35	30.87	1.20	6.11	0.90	2.66	1.889
		58.81	30.76	1.21	5.38	0.87	2.94	1.911
	0.02	56.20	31.63	1.19	7.49	0.90	2.67	1.776
		54.76	33.36	1.35	6.23	1.10	2.99	1.641
		54.25	32.29	1.20	8.68	1.02	2.44	1.680
		57.88	30.74	1.21	6.17	0.96	2.88	1.882
	0.2	57.85	31.40	1.18	5.81	1.26	2.78	1.842
		54.21	35.16	1.57	4.83	1.06	2.82	1.541
		54.58	32.91	1.16	7.93	0.87	2.40	1.658
		54.71	32.91	1.15	7.98	0.82	2.35	1.662
	1.0	57.84	32.00	1.13	5.35	0.86	2.80	1.807
		56.31	32.76	1.06	6.72	0.87	2.50	1.718
		53.86	33.92	1.10	6.75	0.86	3.24	1.587
		55.98	32.77	1.05	6.81	0.83	2.51	1.708
	5.0	55.03	33.39	0.91	7.33	0.74	2.55	1.647
		54.52	31.40	1.03	9.43	0.86	2.82	1.736
		54.20	33.19	0.97	8.01	0.77	2.69	1.632

Acet. = acetic acid, Prop. = propionic acid, I-But. = iso-butyric acid, But. = butyric acid, I-Val. = iso-valeric acid, Val. = valeric acid, Ac/Pr = acetic/propionic acid ratio.

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